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(54) Title: A PROTEIN PREPARATION THAT MEDIATES Ca^{2+}-DEPENDENT TRANSBILAYER MOVEMENT OF PLASMA MEMBRANE PHOSPHOLIPID AND INHIBITORS THEREOF

(57) Abstract

A protein preparation that mediates Ca^{2+}-dependent transbilayer movement of phospholipids is disclosed. A recombinantly engineered DNA sequence encoding the protein, an inhibitor of the protein activity, genetically engineered cells with altered protein activity, and therapeutic methods are also disclosed.

A PROTEIN PREPARATION THAT
MEDIATES Ca²⁺-DEPENDENT TRANSBILAYER MOVEMENT OF
PLASMA MEMBRANE PHOSPHOLIPID AND INHIBITORS THEREOF

BACKGROUND OF THE INVENTION

5   The exposure of phosphatidylserine (PS) and other aminophospholipids (**aminoPL**) on the surface of activated or injured blood cells and endothelium is thought to play a key role in the initiation and regulation of blood coagulation. De novo surface exposure of aminophospholipids has also been

10   implicated in the activation of both complement and coagulation systems after tissue injury, and in removal of injured or apoptotic cells by the reticuloendothelial system. Although migration of these phospholipids (**PL**) from inner-to-outer plasma membrane leaflets is known to be

15   triggered by elevated intracellular [Ca²⁺] ([Ca²⁺]$_i$) and to be associated with vesicular blebbing of the cell surface, little is known about the cellular constituents that participate in this process.

       Role of cell surface PS in coagulation. Several enzyme

20   complexes of the coagulation cascade require assembly on a receptive membrane surface for full expression of catalytic activity (K. G. Mann, et al., Annu. Rev. Biochem. 57:915-956, 1988; S. Krishnaswamy, et al., J. Biol. Chem. 267:26110-26120, 1992; P. B. Tracy, Semin. Thromb. Hemost. 14:227-233, 1988).

25   In the case of the tenase (FVIIIaFIXa) and prothrombinase (FVaFXa) complexes, this surface catalytic function of the plasma membrane is not normally expressed by quiescent cells, but is rapidly induced upon cell activation (in platelets) or upon cell injury (in platelets, endothelium and other cells)

30   (E. M. Bevers, et al., Blood Rev. 5:146-154, 1991; J. Rosing, et al., Blood 65:319-332, 1985; E. M. Bevers, et al., Eur. J. Biochem. 122:429-436, 1982; E. M. Bevers, et al., Biochim.

Biophys. Acta 736:57-66, 1983; T. Wiedmer, *et al., Blood*
68:875-880, 1986). Although specific cell surface protein
receptors for FVa and FVIIIa have been postulated, these
factors show specific avidity for PS-containing liposomes, and

5   in cell-free systems, this lipid alone can support the
catalytic function of the prothrombinase and tenase enzymes
(J. Rosing, *et al., supra,* 1985; M. E. Jones, *et al., Thromb.*
Res. 39:711-724, 1985; G. E. Gilbert, *et al., Biochemistry*
32:9577-9585, 1993; G. E. Gilbert, *et al., J. Biol. Chem.*

10   265:815-822, 1990; G. E. Gilbert, *et al., J. Biol. Chem.*
267:15861-15868, 1992). We and others have shown that PS
rapidly moves to the surface of the plasma membrane upon
platelet stimulation, and that this exposure of PS correlates
with expression of the platelet's FVa & FVIIIa binding sites

15   and expression of surface catalytic function for tenase and
prothrombinase (P. Williamson, *et al., Biochemistry*
31:6355-6360, 1992; F. Bassé, *et al., Biochemistry*
32:2337-2344, 1993; C.-P. Chang, *et al., J. Biol. Chem.*
268:7171-7178, 1993; J. Connor, *et al., Biochim. Biophys.*

20   Acta1025:82-86, 1990; P. Comfurius, *et al., Biochim. Biophys.*
Acta 1026:153-160, 1990). Smeets, *et al., Biochim. Biophys.*
Acta 1195:281-286, 1994, Williamson, *et al., Biochem.*
34:10448-10455, 1995; Bratton D.L., *J. Biol. Chem.*
269:22517-22523, 1994). Additional evidence that

25   surface-exposed PS provides the physiological receptor site
for these enzyme complexes is provided by (1) the capacity of
PS-containing liposomes or phosphoserine to compete binding of
FVIIIa to activated platelets (G.E. Gilbert, *et al., J. Biol.*
Chem. 266:17261-17268, 1991), (2) the capacity of annexin V

30   and other proteins with affinity for membrane PS to mask the
FVa and FVIIIa binding sites expressed by activated platelets
(P. Thiagarajan, *et al., J. Biol. Chem.* 265:17420-17423, 1990;
P. Thiagarajan, *et al., J. Biol. Chem.* 266:24302-24307, 1991;
J. Dachary-Prigent, *et al., Blood* 81:2554-2565, 1993; J. Sun,

35   *et al., Thromb. Res.* 69:289-296, 1993); (3) evidence that
platelets congenitally deficient in inducible FVa and FVIIIa
receptors are also defective in stimulated exposure of plasma
membrane PS ("Scott syndrome"; *see below*) (J. P. Miletich, *et*

al., *Blood* 54:1015-1022, 1979; J. Rosing, *et al.*, *Blood*
65:1557-1561, 1985; P. J. Sims, *et al.*, *J. Biol. Chem.*
264:17049-17057, 1989; S. S. Ahmad, *et al.*, *J. Clin. Invest.*
84:824-828, 1989; F. Toti, *et al.*, *Blood* 87:1409-1415, 1996).

5   In addition to the catalytic function PS provides to the
prothrombinase and tenase complexes, surface exposed
aminophospholipids have been shown to promote the activities
of the tissue factor-FVIIa and protein S-activated protein C
enzyme complexes of the coagulation system , as well as the

10   activity of the alternative pathway C3-convertase (C3bBb
enzyme complex) of the complement system (W. Ruf, *et al.*, *J.
Cell. Biol.* 266:2158-2166, 1991; F. J. Walker, *J. Biol. Chem.*
256:11128-11131, 1981; R. H. Wang, *et al.*, *J. Clin. Invest.*
92:1326-1335, 1993; P. F. Neuenschwander, *et al.*, *Biochemistry*

15   34:13988-13993, 1995).

In addition to the central role that inducible expression
of plasma membrane PS is thought to play in the platelet
hemostatic response, the surface exposure of PS and
phosphatidylethanolamine (PE) in response to membrane injury

20   has been implicated in a variety of thrombotic and
inflammatory disorders. For example, repeatedly sickled SS
hemoglobin erythrocytes exhibit increased surface exposure of
PS, which promotes prothrombinase assembly and accelerates
plasma clotting *in vitro*, and may contribute to thrombotic

25   complications that can arise in sickle cell disease (P. F.
Franck, *et al.*, *J. Clin. Invest.* 75:183-190, 1985; N.
Blumenfeld, *et al.*, *Blood* 77:849-854, 1991). Increased PE
exposure on sickled RBCs (and other cells) has also been shown
to promote complement activation with resulting accumulation

30   of C3b/C3d and C5b-9 on the cell surface, potential factors
contributing to the accelerated clearance and increased
fragility of these cells (R. H. Wang, *et al.*, *supra*, 1993).
PS exposure secondary to immune injury to the endothelium has
also been implicated in the thrombo-embolic complications of

35   hyperacute graft rejection, and PS exposure secondary to C5b-9
accumulation on platelets and red cells has been suggested to
contribute to the high risk of venous thrombosis in Paroxysmal
Nocturnal Hemoglobinuria (J. L. Platt, *et al.*, *Immunol. Today*

11:450-6; discuss, 1990; A. P. Dalmasso, *Immunopharmacology*
24:149-160, 1992; A. P. Dalmasso, *et al.*, *Am. J. Pathol.*
140:1157-1166, 1992; T. Wiedmer, *et al.*, *Blood* 82:1192-1196,
1993, K. K. Hamilton, *et al.*, *J. Biol. Chem.* 265:3803-3814,
5   1990; S. P. Kennedy, *et al.*, *Transplantation* 57:1494-1501,
1994)). In the "antiphospholipid syndromes," the interaction
of exposed plasma membrane PS and PE with plasma proteins is
now generally believed to induce offending antigens (M.D.
Smirnov, *et al.*, *J. Clin. Invest.* 95: 309-316, 1995).

10   **Relationship of PS exposure to programmed cell death.**
Programmed cell death (apoptosis) is now recognized to be
central to the selective elimination of mammalian cells during
embryogenesis, tissue re-modeling, and in the clonal selection
of immune cells (P. D. Allen, *et al.*, *Blood Rev.* 7:63-73,
15   1993; J. J. Cohen, *Immunol. Today* 14:126-130, 1993). The
apoptotic cell undergoes characteristic changes, including
elevated $[Ca^{2+}]_i$, altered phospholipid packing, surface
exposure of PS, plasma membrane blebbing and vesiculation,
cell shrinkage, chromatin condensation, nucleolar
20   desintegration, and at late stages, DNA degradation by
Ca^{2+}/Mg^{2+}-dependent endonuclease(s), with characteristic
fragmentation into 180 bp multimers ("DNA laddering"). The
transcriptional events that initiate apoptosis remain
unresolved, but evidence implicates certain proto-oncogenes,
25   including c-myc as activators, and other proto-oncogenes,
including bcl-2, as suppressors (P. D. Allen, *et al.*, *supra*,
1993; J. C. Reed, *J. Cell. Biol.* 124:1-6, 1994). In
thymocytes and B-lymphocytes, an apoptotic transformation can
be induced by dexamethasone (activating glucocorticoid
30   receptors) and by cAMP (protein kinase A pathway) (D. J.
McConkey, *et al.*, *J. Immunol.* 145:1227-1230, 1990; N. Kaiser,
et al., *Proc. Natl. Acad. Sci. USA* 74:638-642, 1977; J. J.
Cohen, *et al.*, *J. Immunol.* 132:38-42, 1984; R. Merino, *et al.*,
EMBO J. 13:683-691, 1994; M. K. Newell, *et al.*, *Proc. Natl.*
35   *Acad. Sci. USA* 90:10459-10463, 1993), as well as directly
through treatment with Ca^{2+} ionophore (Z.-Q. Ning, *et al.*, *Eur.*
J. Immunol. 23:3369-3372, 1993), implicating $[Ca^{2+}]_i$ as a

central mediator of the cellular changes that accompany
apoptosis. The similarity of the plasma membrane changes
noted for apoptotic cells, to those elicited by elevation of
$[Ca^{2+}]_i$ in platelets, erythrocytes, and other cells that do not
5   undergo apoptosis, suggest that the nuclear and plasma
membrane changes associated with apoptosis are separate
"epiphenomena", reflecting independent and unrelated responses
to a coordinate rise in $[Ca^{2+}]_i$.

Diaz, et al. (Blood 87[7]:2956-2961, 1996) have recently
10   reported the generation of phenotypically aged
phosphatidylserine-expressing erythrocytes by
dilauroylphosphatidylcholine (DLPC)-induced vesiculation. Red
blood cells were artificially vesiculated with DLPC and
assessed for alterations in density, membrane lipid asymmetry
15   and propensity to be recognized by macrophages in vitro and
the reticuloendothelial system in vivo. The results suggest
that vesiculation contributes to alterations is membrane lipid
asymmetry and cell characteristics of the aged red blood cell
phenotype.

20   **Role of cell surface PS in clearance by the RE system.**
There is now accumulating data to suggest that cell-surface PS
may contribute to the recognition and clearance of senescent,
injured, or apoptotic cells by macrophages and other cells of
the reticuloendothelial system (J. Savill, et al., Immunol.
25   Today 14:131-136, 1993; V. A. Fadok, et al., J. Immunol.
148:2207-2216, 1992; J. Connor, et al., J. Biol. Chem.
269:2399-2404, 1994). These experiments demonstrate that (i)
macrophages have inducible receptors that stereospecifically
bind to PS-containing liposomes and to surface-exposed plasma
30   membrane PS; (ii) selective phagocytosis of apoptotic
lymphocytes by stimulated macrophages is observed in the
absence of plasma proteins, and this can be inhibited by
PS-containing liposomes or by phosphoserine (V. A. Fadok, et
al., supra, 1992). Consistent with these data, the
35   circulating lifetime of infused PS-containing liposomes is
markedly decreased when compared to liposomes devoid of PS,
due to rapid hepato-splenic clearance (T. M. Allen, et al.,

Proc. Natl. Acad. Sci. USA 85:8067-8071, 1988). Similarly,
increased exposure of plasma membrane PS during *in vitro*
storage of platelet concentrates may contribute to an
accelerated clearance of these cells after transfusion (A. P.

5   Bode, et al., *Thromb. Res.* 39:49-61, 1985; A. P. Bode, et al.,
J. Lab. Clin. Med. 113:94-102, 1989; A. P. Bode, et al., *Blood*
77:887-895, 1991; D. Geldwerth, et al., *J. Clin. Invest.*
92:308-314, 1993; P. Gaffet, et al., *Eur. J. Biochem.*
222:1033-1040, 1994; E. M. Bruckheimer, et al., *J. Leukoc.*

10   *Biol.* 59, 784-788, 1996; C. Diaz, et al, *supra*, 1996). This
possibility is underscored by recent reports documenting
increased PS exposure in platelets and red cells during *in
vitro* storage. Evidence that PS exposed on the surface of
tumor cells promotes adherence and cytolysis by inflammatory

15   macrophages has also been reported (J. Connor, et al., *Proc.
Natl. Acad. Sci. USA* 86:3184-3188, 1989).

      Regulation of the transmembrane distribution of PS. It
is now well established that phospholipids are normally
asymmetrically distributed within the plasma membrane of all

20   blood cells, vascular endothelium, and other cells: the
aminophospholipids (including phosphatidylserine (**PS**) and
phosphatidylethanolamine (**PE**)) reside almost exclusively in
the inner membrane leaflet, whereas the outer leaflet is
enriched in neutral polar phospholipids, including

25   phosphatidylcholine (**PC**) and sphingomyelin (B. Roelofsen,
Infection 19:S206-S209, 1992; A. J. Schroit, et al., *Biochim.
Biophys. Acta* 1071:313-329, 1991; P. F. Devaux, *Biochemistry*
30:1163-1173, 1991). It is well-recognized that the
transmembrane orientation of plasma membrane PL is central to

30   the regulation of surface-localized enzyme reactions of both
complement and coagulation systems and to the recognition and
phagocytic clearance of injured, aged or apoptotic cells. It
is also now generally accepted that the maintenance of PL
asymmetry arises through the activity of a specific

35   transmembrane PL "flippase" with specificity for aminoPL.
This aminoPL translocase (**APT**) has been shown to selectively
and vectorially transport PS (> PE), but not neutral PL such

as PC, from outer to inner leaflets of the plasma membrane in
a process that is dependent on both Mg^{2+} and ATP, inhibited by
fluoride, o-vanadate or increased $[Ca^{2+}]_c$, and inactivated by
N-ethylmaleimide (NEM) or pyridyldithioethylamine (PDA) (M.

5   Bitbol, et al., Biochim. Biophys. Acta 904:268-282, 1987; M.
Seigneuret, et al., Proc. Natl. Acad. Sci. USA 81:3751-3755,
1984; J. Connor, et al., Biochemistry 26:5099-5105, 1987; P.
F. Devaux, et al., Phys. Lipids 73:107-120, 1994; A.
Zachowski, et al., Biochemistry 25:2585-2590, 1986; C. Diaz,

10   et al., supra, 1996). In addition to plasma membrane, APT
activity has also been identified in the membranes of
secretory vesicles and synaptosomes (A. Zachowski, et al.,
Nature 340:75-76, 1989). The K_m for ATP is approximately 1
mM, and it has been estimated that one molecule of ATP is

15   hydrolyzed for each aminoPL transported (Z. Beleznay, et al.,
Biochemistry 32:3146-3152, 1993). Two candidate proteins have
been proposed to function as APT: the Rh antigen protein, and
a 110-120 kDa Mg^{2+}-ATPase. Schroit and coworkers (A. J.
Schroit, et al., Biochemistry 29:10303-10306, 1990) originally

20   proposed that a 32 kDa PS-binding RBC membrane protein that
precipitated with antibody to Rh was the erythrocyte APT.
Subsequently it was shown that Rh_{null} cells deficient in Rh
antigen nevertheless exhibit normal APT activity, and very
recently, the 32 kDa PS-binding protein that co-precipitates

25   with Rh protein was identified as stomatin, and it was shown
that its interaction with PS was not specific for the aminoPL
headgroup. This implies that neither stomatin, nor, the Rh
protein can provide APT function (J. Desneves, et al.,
Biochem. Biophys. Res. Commun. 224:108-114, 1996). The

30   observed similarity in cation, ATP- and PS-dependence of
cellular APT activity to a partially purified Mg^{2+}-dependent
ATPase from RBC led Devaux and associates, and later Daleke,
to suggest that APT is a specific Mg^{2+}-ATPase (A. Zachowski,
et al.supra, 1989; G. Morrot, et al., FEBS Lett. 266:29-32; D.

35   L. Daleke, et al., Ann. NY Acad. Sci. 671:468-470, 1992; M. L.
Zimmerman, et al., Biochemistry 32:12257-12263, 1993).
Consistent with this premise, Auland (Auland, et al., Proc.
Natl. Acad. Sci 91:10938-10942, 1994) demonstrated PS-specific

transport in proteoliposomes reconstituted with an unidentified Mg^{2+}-ATPase isolated from RBC. Recently, an ATPase II from bovine chromaffine granules has been cloned and sequenced, and evidence has been presented that this enzyme
5   may exhibit aminoPL translocase activity (X. J. Tang, et al., Science 272:1495-1497, 1996).

Ca^{2+} and the collapse of phospholipid asymmetry

Whereas the rate of spontaneous flip/flop of PL between membrane leaflets is normally quite slow, a substantial rise
10 in $[Ca^{2+}]_c$ resulting from agonist-induced activation, programmed cell death, or, secondary to immune injury, initiates rapid transbilayer migration of all plasma membrane PL with net movement of aminoPL to the outer leaflet, collapsing the normal asymmetric distribution (P. Williamson,
15 et al., Biochemistry 31:6355-6360, 1992); F. Bassé, et al., Biochemistry 32:2337-2344, 1993; C.-P. Chang, et al., supra, 1993; P. Comfurius, et al. Biochim. Biophys. Acta 1026:153-160 1990; A. J. Schroit, supra, 1991; P. Devaux, Biochemistry 30:1163-1173, 1991; J. Connor, et al., J. Biol. Chem.
20 267:19412-19417, 1992). Four different mechanisms have been proposed to account for this induced "scrambling" of plasma membrane PL with net egress of aminoPL to cell surfaces (A. J. Schroit, supra, 1991; P. Devaux, supra, 1992; P. Devaux, supra, 1991; R. F. A. Zwaal, et al., Biochim. Biophys. Acta
25 1180:1-8, 1992): (i) spontaneous collapse of PL asymmetry due to inactivation of vectorial transport by plasma membrane APT; (ii) random scrambling due to transient formation of non-bilayer (H_{II}-phase) PL domains upon Ca^{2+}-induced blebbing of plasma membrane vesicles; (iii) direct effects of Ca^{2+} on
30 topology and distribution of anionic PLs; (iv) response of a Ca^{2+}-sensitive protein(s) that facilitates PL transfer between membrane leaflets.

(i) Spontaneous collapse of PL asymmetry. APT is inhibited at elevated $[Ca^{2+}]_c$, raising the possibility that the concomitant
35 egress of aminoPL to the cell surface simply reflects spontaneous back-leak ("flop") of the PL distribution that is constitutively maintained by APT (P. Williamson, et al.,

supra, 1992; P. F. Devaux, *supra*, 1992; P. Devaux, *supra*,
1991). Nevertheless, inhibition of APT--either by depletion
of cellular ATP, or by incubation with fluoride, o-vanadate or
NEM--does not in itself cause accelerated transbilayer PL

5 migration or significant cell-surface PS exposure, as long as
normally low $[Ca^{2+}]_c$ is maintained (E. M. Bevers, *et al.*,
supra, 1991; P. Comfurius, *et al.* *supra*, 1990; B. Verhoven, *et
al.*, *Biochim. Biophys. Acta* 1104:15-23, 1992; J. Connor, *et
al.* *Biochemistry* 29:37-43, 1990). Conversely, cells

10 genetically deficient in PL scramblase show normal APT
activity. Thus it appears that the spontaneous transbilayer
migration of plasma membrane PL is inherently very slow at the
normal low $[Ca^{2+}]_c$, whereas entry of Ca^{2+} into the cytosol
specifically induces rapid movement of PL between plasma

15 membrane leaflets. Whereas the inherent rate of transbilayer
migration of PL cannot account for the rapid scrambling
observed at elevated $[Ca^{2+}]_c$, it is conceivable that
interaction of $[Ca^{2+}]_c$ with APT induces a conformational change
that not only inactivates the ATP-dependent inward

20 translocation of aminoPL, but also facilitates selective flop
of PS & PE to the outer leaflet. In this context, Bienvenue
and associates (Bassé, *et al*, *supra*, 1993; Gaffet, *et al.*,
Biochemistry 34:6762-6769, 1995) have reported evidence for
transient vectorial egress of PS upon elevation of $[Ca^{2+}]_c$ in

25 platelet, whereas data from others suggest bidirectional and
non-selective transbilayer scrambling of all plasma membrane
PL (including PC which is not flipped by APT) at elevated
$[Ca^{2+}]_c$ (P. Williamson, *et al.*, *supra*, 1992; P. Williamson, *et
al.*, Biochemistry 34:10448-10455, 1995; E. F. Smeets, et al.,

30 Biochim. Biophys. Acta Bio-Membr. 1195:281-286, 1994; D.
Bratton, J. Biol. Chem. 269:22517-22523, 1994).

(ii) **Relationship of PS egress to shedding of plasma membrane
vesicles.** Data from our laboratory helped establish that
surface exposure of PS is intimately related to a process of

35 Ca^{2+}-induced vesiculation of the plasma membrane, and that
formation of such PS-rich plasma membrane "microparticles"
contributes to expression of cellular procoagulant activity
(C.-P. Chang, *et al.*, *supra*, 1993; P. J. Sims, *et al.*, *supra*,

1989; A. P. Bode, *et al.*, *supra*, 1985; R. F. A. Zwaal, *et al.*, *supra*, 1992; T. Wiedmer, *et al.*, *supra*, 1990; P. J. Sims, *et al.*, *J. Biol. Chem.* 263:18205-18212, 1988; K. K. Hamilton, *et al.*, *J. Biol. Chem.* 265:3809-3814, 1990; H. Sandberg, *et al.*,

5   *Thromb. Res.* 39:63-79, 1985). This correlation between microparticle formation and surface exposure of PS suggested that the membrane fusion events generating these membrane vesicles underlie observed scrambling of plasma membrane PL, presumably through transient formation of H_{II}-phase PL (C. P.

10   Chang, *et al.*, *supra*, 1993). Alternatively, prior egress of PS to the outer leaflet might create a mass imbalance that itself drives plasma membrane evagination and vesiculation (P.F. Devaux, *supra*, 1991). In this context, we and others have observed that PS migration to the cell surface can

15   precede membrane vesiculation, and can occur without microparticle formation (G. E. Gilbert, *et al.*, *J. Biol. Chem* 266:17261-18269, 1991; F. Basse, *et al.*, *Biochemistry* 32:2337-2344, 1993; P. Gaffet, *et al.*, *supra*, 1995)). Our data suggested the participation of a calmodulin-dependent kinase

20   in Ca^{2+}-induced vesiculation (T. Wiedmer, *et al.*, *Blood* 78:2880-2886, 1991). Involvement of a protein kinase in the cytoskeletal reorganization required for platelet microparticle formation has recently been confirmed (Y. Yano, *et al. Biochem. J.* 298:303-308, 1994). This suggests that

25   whereas membrane fusion may contribute, it neither initiates nor is required for PL scramblase function.
     (iii) Interaction of Ca^{2+} with anionic plasma membrane PL.
     Ion pairing of Ca^{2+} with the anionic PS headgroup might accelerate transbilayer migration by lowering the energy

30   barrier to crossing through the hydrophobic membrane interior. However, Ca^{2+} does not directly induce transbilayer migration of PL in PS-containing membranes, except when mole% of PS is sufficient to induce an H_{II}-phase and vesicle-vesicle fusion (A.L. Bailey, et al., Biochemistry 33:12573-12580, 1994; B. de

35   Kruijff, et al., Trends Biochem. Sci. 5:79-81, 1980). Thus ion pairing of Ca^{2+} with inner leaflet PS would not appear to directly induce PL flip/flop between membrane leaflets. Alternatively, Devaux and associates (J.-C. Sulpice, *et al.*,

J. Biol. Chem. 269:6347-6354, 1994; J.-C. Sulpice, et al.,
Biochemistry 35:13345-13352, 1996) proposed that Ca^{2+} binding
to phosphatidylinositol 4,5-bisphosphate (PIP2) induces
transbilayer migration of other PL, based on the observation

5   that adding PIP2 to RBC promoted Ca^{2+}-dependent transbilayer
movement of PS. Nevertheless, subsequent studies revealed
that this incorporation of exogenous PIP2 also induces enough
membrane lysis to account for any apparent increase in
transbilayer migration of PS (Bevers, et al. Blood

10   86:1983-1991, 1995).

(iv) **Role of Ca^{2+}-sensitive protein(s) in PL redistribution.**
Elevation of $[Ca^{2+}]_c$ is known to give rise to marked changes in
several cytoskeletal and membrane proteins that might affect
the rate of movement of PL between plasma membrane leaflets.

15   For example, several cytoskeletal proteins (including,
spectrin and erythrocyte band 4.1)(A. M. Cohen, et al., Blood
68:920-926, 1986; S. B. Sato, et al., Eur. J. Biochem.
130:19-25, 1983; A. C. Rybicki, et al., J. Clin. Invest.
81:255-260, 1988; K. A. Shiffer, et al., Biochim. Biophys.

20   Acta 937:269-280, 1988) have been shown to bind specifically
to the PS headgroup, and this interaction potentially serves
to trap PS in the inner leaflet of the plasma membrane (P.
Comfurius, et al., Biochim. Biophys. Acta 983:212-216, 1989).
Breaking such interactions, as might occur through interaction

25   of Ca^{2+} with the PS headgroup, or through proteolytic
degradation of cytoskeletal proteins, would potentially
dissociate PS from these endofacial contacts and thereby
accelerate PS flop to the outer leaflet (P. F. Franck, et al.,
supra, 1985). In this context, it has been reported that

30   polyamines inhibit the endogenous PL scramblase activity of
the RBC membrane, suggesting that these polycations inhibit
interaction of Ca^{2+} at its endofacial membrane site (D. L.
Bratton, et al., supra, 1994; J.-C. Sulpice, et al. supra,
1996). Calpain-mediated proteolysis of components of the

35   submembrane cytoskeleton can be temporally-correlated to
membrane vesiculation and to surface exposure of PS.
Nevertheless, inhibition of calpain does not prevent PS
egress, and, Ca^{2+}-dependent PL scramblase activity is readily

demonstrated in resealed RBC ghosts and inside-out RBC
membrane vesicles (IOV) washed free of calpains and other
soluble proteases (P. Comfurius, et al., *supra*, 1990; T.
Wiedmer, et al., *Biochemistry* 29:623-632, 1990; ·Fox, et al.,

5   *J. Biol. Chem.* 266:13289-13295, 1991; J. E. B. Fox, et al., *J.
Cell Biol.* 111:483-493, 1990; P. F. J. Verhallen, et al.,
Biochim. Biophys. Acta 903:206-217, 1987; L. Dachary-Prigent,
et al., *Blood* 81:2554-2565, 1993)   On the other hand, the
possibility that a membrane protein with inherent PL

10   scramblase activity directly mediates Ca^{2+}-induced
transbilayer migration of PL was suggested by the observation
that this activity is inhibited by sulfhydryl oxidation of
membrane proteins with PDA (P. Williamson, et al., *supra*,
1995).   Consistent with this finding, we recently reported the

15   purification and preliminary characterization of an integral
RBC membrane protein that, when reconstituted in liposomes,
mediates a Ca^{2+}-dependent transbilayer movement of PL
mimicking plasma membrane PL reorganization evoked upon
elevation of $[Ca^{2+}]_c$ (F. Bassé, et al., *J. Biol. Chem.*

20   271:17205-17210, 1996) Evidence that a protein of similar
function must also be present in platelets was recently
reported by Zwaal (P. Comfurius, et al., *Biochemistry*
35:7631-7634, 1996).

    The Scott syndrome. Scott syndrome is a bleeding

25   disorder described in a single patient that reflects impaired
expression by activated platelets of the membrane sites that
are required for normal assembly of the prothrombinase and
tenase enzyme complexes (H. J. Weiss, et al., *Am. J. Med.*
67:206-213, 1979; H. J. Weiss, *Semin. Hematol.* 31:1-8, 1994).

30   Platelets obtained from this patient secrete and aggregate
normally when exposed to various agonists (ADP, thrombin,
collagen, complement proteins C5b-9, or calcium ionophore),
but when fully activated, exhibit a marked deficiency of
membrane binding sites for factors Va and VIIIa, reflecting a

35   concomitant reduction in the amount of surface-exposed PS (J.
P. Miletich, et al., *supra*, 1979; J. Rosing, et al., *supra*,

1985; J. P. Sims, *et al.*, *supra*, 1989; S. S. Ahmad, *et al.*, *supra*, 1989).

Although Scott syndrome was originally described as an isolated platelet disorder, it is now clear that other blood
5   cells from this patient, including erythrocytes and lymphocytes, are affected as well (E. M. Bevers, *et al.* *Blood* 79:380-388, 1992). Measurements that have been made by ourselves and others suggest that Scott platelets and erythrocytes contain normal amounts of PS and other
10 phospholipids, and also exhibit normal aminophospholipid translocase activity (H. J. Weiss, *et al.*, 1979). These cells are defective, however, in their capacity to mobilize PS from inner to outer membrane leaflets in response to elevated $[Ca^{2+}]_i$, a response that is now thought to be required for
15 normal prothrombinase and tenase assembly. A search for the molecular defect responsible for the Scott syndrome has to date failed to reveal abnormality in platelet or red cell proteins (probed by 2-dimensional electrophoresis); the agonist-induced elevation of $[Ca^{2+}]_i$ in Scott platelets is
20 normal; and the calpain and transglutaminase activities of both Scott platelets and RBCs are indistinguishable from normal controls, as assessed by the Ca^{2+}-induced cleavage or cross-linking of cytoskeletal proteins (P. Comfurius, *et al.*, *Biochim. Biophys. Acta* 815:143-148, 1985).

25 We have established in *in vitro* culture EBV-transformed lymphoblast cell lines from this patient and have demonstrated that these immortalized cells exhibit the same phenotype of impaired Ca^{2+}-induced plasma membrane phospholipid scrambling that is characteristic of the platelets and erythrocytes in
30 this disorder. Our data also establish that this defect propagates through multiple cell divisions and can be corrected by heterokaryon fusion with wild-type cells that exhibit normal plasma membrane phospholipid scrambling (H. Kojima, *et al.* *J. Clin. Invest.* 94:2237-2244, 1994).

35 Similar data have recently been reported for a second patient with Scott syndrome, and evidence for a familial inheritance of the cellular defect provided (F. Toti, *et al.*, *supra*, 1996)). This implies that the molecular basis for this

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clinical disorder relates to a gene defect that results in impaired activity of a cellular protein that is expressed in a variety of cell lineages, and that this protein normally mediates the intracellular Ca^{2+}-dependent transbilayer

5   movement (or "scrambling") of plasma membrane phospholipids that occurs in response to cell activation, apoptosis, or cell injury. We identify this protein as "phospholipid scramblase", "PL scramblase", and "P37". We mean for "P37" to be synonymous with "phospholipid scramblase or PL scramblase" and

10   refer to these names interchangeably throughout the text.

The loss of normal PL scramblase function in patients with Scott syndrome may relate to either the abnormal expression of an inhibitor of the activity of PL scramblase, a deletion or loss of function mutation in the gene encoding P37

15   protein, or, a mutation affecting a cofactor of P37 that is required for normal expression of its PL scramblase activity.

Patients with the Scott syndrome defect display abnormal bleeding and a prolongation of the time required for blood clotting (H. J. Weiss, *Semin. Hematol.* 31:1-8, 1994). This

20   implies that activation of PL scramblase is normally required for effective clot formation and for efficient hemostasis, whereas loss or inhibition of PL scramblase activity leads to retarded blood clotting. We therefore propose that the selective *activation* of PL scramblase function is of potential

25   therapeutic value in the acceleration of hemostasis and in preventing blood loss, whereas the selective *inhibition* of PL scramblase function is of potential therapeutic value in certain thrombotic disorders characterized by excessive or inappropriate clot formation due to expression of plasma

30   membrane procoagulant activity.

In this application, we identify the cellular component that functions to mediate the Ca^{2+}-dependent reorganization of plasma membrane phospholipids and describe methods for preventing egress of PS to the surface of activated, injured,

35   or apoptotic cells.

BRIEF SUMMARY OF THE INVENTION

The present invention relates to the creation and use of
antithrombotic and thrombostatic reagents that depend on the
properties of a protein preparation that mediates Ca^{2+}
5   dependent transbilayer movement of membrane phospholipids.

The present invention is a preparation of a protein,
wherein the protein is a phospholipid scramblase and wherein
the protein is approximately 35-37 kD as measured on a 12.5%
SDS-polyacrylamide gel under reducing conditions. Preferably,
10   the scramblase comprises residues 59 - 90 of SEQ ID NO:1.
More preferably, the preparation comprises residues 1 - 188 of
SEQ ID NO:1.

In another embodiment of the present invention, the PL
scramblase comprises residues 59 - 90 of SEQ ID NO:1 with
15   conservative or functionally equivalent substitutions.

In the most preferred embodiment of the present
invention, the PL scramblase comprises residues 75-392 of SEQ
ID NO:4 with conservative or functionally equivalent
substitutions.

20   The present invention is also a DNA sequence encoding the
PL scramblase. Preferably, this DNA sequence comprises the
nucleic acids 223-1176 of SEQ ID NO:3.

The present invention is also an inhibitor of the PL
scramblase activity of P37. This inhibitor may be an
25   antisense nucleotide derived from the DNA sequence of P37. In
another embodiment, the inhibitor is a peptide sequence that
is a competitive inhibitor of P37 PL scramblase activity.
Preferably, the inhibitor is a peptide that either binds to
plasma membrane phosphatidylserine, prevents binding of Ca^{2+}
30   to P37, or prevents its phosphorylation by cellular protein
kinases. In another embodiment, the inhibitor is an antibody,
preferably a monoclonal antibody, raised against P37.

The present invention is also a method for preventing the
surface exposure of plasma membrane phosphatidylserine,
35   phosphatidylethanolamine and cardiolipin on the surface of *in
vitro* stored platelets or red blood cells. This method
comprises the steps of adding an inhibitor of P37 PL

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scramblase activity to the stored platelets or red blood
cells.

The present invention is also a method for prolonging
survival of transplanted organs comprising the step of adding
5   an inhibitor of P37 PL scramblase activity to an organ
perfusate during in vitro organ storage. The present invention
is also a method for prolonging the survival of transplanted
cells, tissues, and organs by genetically engineering the
cells to be transplanted so as to alter their expression of
10   plasma membrane P37 in order to reduce exposure of PS and
other thrombogenic phospholipids at the plasma membrane
surface, thereby reducing the risk of infarction due to fibrin
clot formation.

The present invention is also a method for prolonging the
15   in vivo survival of circulating blood cells (erythrocyte,
platelets, lymphocyte, PMN's, and monocytes) comprising the
step of preventing surface exposure of plasma membrane
phosphatidylserine on the surface of the cells by exposing the
blood cells to an inhibitor of P37 PL scramblase activity.

20   The present invention is also a method for preventing the
procoagulant activities of erythrocytes in sickle cell disease
comprising the step of inhibiting erythrocyte P37 in a sickle
cell patient.

The present invention is also a method for treating
25   autoimmune and inflammatory diseases comprising the step of
treating a patient with an inhibitor of the PL scramblase
activity of P37.

The present invention is also a method for diagnosing
individuals with reduced or elevated capacity for
30   platelet-promoted or erythrocyte-promoted fibrin clot activity
comprising the step of quantitating the cellular expression of
P37. This quantitation may take the form of immunoblotting
using an antibody to P37, an ELISA assay using an antibody to
P37, flow cytometric analysis of the binding of monoclonal
35   antibody reactive against the predicted extracellular domain
of P37 (residues 386 - 392 of sequence disclosed in SEQ ID
NO:4) or using oligonucleotides derived from P37 cDNA and the
polymerase chain reaction. In one method of the present

invention, the quantitation is performed by isolating P37 from
a patient blood sample, measuring the amount of P37 isolated
and comparing the measurement with a control sample. The
measurement may be by isolating P37 from a patient blood
5   sample and measuring via densitometry the amount of P37
protein electrophoresed in a stained electrophoretic gel.

It is an object of the present invention to provide a
preparation of a PL scramblase.

It is another object of the present invention to provide
10   an inhibitor of P37 PL scramblase activity.

It is another object of the present invention to provide
an antithrombotic agent.

It is another object of the present invention to create
cells, tissue, and organs for transplantation that have
15   increased potential for survival and reduced potential for
causing fibrin clot formation and vascular thrombosis when
grafted into a recipient host.

Other objects, advantages and features of the present
invention will become apparent after one of skill in the art
20   reviews the specification, claims and drawing herein.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1A is a bar graph depicting the amount of PL
scramblase activity as a function of chromatography column
fraction number. Fig. 1B represents a 12.5% SDS-PAGE in which
25   the fractions described in Fig. 1A have been electrophoresed.
The actual 37 kD bands are depicted by dark rectangles.

Fig. 2 is a diagram of the bidirectional movement of
NBD-PS across lipid bilayers reconstituted with P37.

Fig. 3 is a graph describing the kinetics of PS
30   translocation by reconstituted P37.

Fig. 4 is a graph describing calcium dependence of P37
activity in proteoliposomes and in erythrocyte IOVs.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is the purification and
characterization of an approximately 35-37 kD membrane protein
from human erythrocytes that promotes a Ca^{2+}-dependent
5   transbilayer redistribution of membrane phospholipids
including PS and PC, with properties similar to the PL
scramblase activity that is evoked upon elevation of Ca^{2+} in
the cytosol of erythrocytes and other cells. We have named
this membrane protein "P37." We mean for "P37" to be
10   synonymous with "phospholipid scramblase or PL scramblase" and
refer to these names interchangeably throughout the text. By
"phospholipid scramblase or PL scramblase activity," we mean
the Ca^{2+} dependent transbilayer movement of plasma membrane
phospholipid.

15   In one embodiment, the present invention is a protein
preparation of P37. Preferably, the protein comprises
residues 59 - 90 of SEQ ID NO:1. More preferably, the PL
scramblase comprises residues 1 - 188 of SEQ ID NO:1. Most
preferably, the protein comprises residues 75 - 392 of SEQ ID
20   NO:4.

In another embodiment, the protein comprises conservative
substitutions or functionally equivalent residues of the
residues described in the paragraph above. By "functionally
equivalent" we mean that the equivalent residues do not
25   inhibit or disrupt the activity of the PL scramblase
preparation.

The examples below demonstrate one method of isolating
P37 from human erythrocytes. After examination of the
specification below, other methods of protein isolation will
30   become apparent to one of skill in the art. The examples
below also describe an assay for the measurement of PL
scramblase activity. A suitable preparation of the present
invention would have a PL scramblase activity of at least 10%
that of the preparation described below in the examples.
35   Preferably, the activity would be at least 50% that of the
examples described below.

The present invention is also a recombinant DNA sequence
encoding P37. A preferable DNA sequence encoding P37 would

comprise the nucleic acids of SEQ ID NO:2. A more preferable
DNA sequence encoding P37 would comprise the nucleic acids 223
- 1176 of SEQ ID NO:3. One of skill in the art of molecular
biology would know how to obtain DNA sequences encoding the PL
5   scramblase. For example, one might sequence P37 directly via
standard protein sequencing techniques as we report below in
Example C. The peptide sequence could be analyzed to provide
oligonucleotide probes for a human cDNA leukocyte library.
(One such cDNA library is available from Invitrogen in a
10 pCDNA3 vector.)

        Residues 59 - 90 of SEQ ID NO:1 are the amino acid
sequence that we obtained of a cyanogen bromide fragment of
the PL scramblase. SEQ ID NO:1 is the translated product of
ATCC clone 962235 and is a longer amino acid sequence
15 containing residues 59 - 90. Residues 75 - 392 of SEQ ID NO:4
is the predicted full-length sequence of the P37 polypeptide,
based on analysis of the open reading frame of SEQ ID NO:3.

        By use of probes obtained from other domains of the PL
scramblase one would then be able to isolate a cDNA clone
20 encoding the entire P37 protein sequence. Sequence identified
as SEQ ID NO:3 contains the entire open reading frame encoding
P37 as well as flanking residues of 5' and 3' untranslated
sequence. The full-length translation of SEQ ID NO:3 is
identified as SEQ ID NO:4. (The symbol Xaa indicates a stop.)
25 Analysis of the open reading frame of this sequence identifies
residues 75 - 392 of SEQ ID NO:4 as comprising the full-length
protein sequence of P37. No signal peptide sequence is
identified between residues 75 -392 of SEQ ID NO:4, indicating
that this is also likely to be the length of the mature
30 polypeptide after post-translational processing. The
calculated molecular weight of the polypeptide containing
residues 75-392 of SEQ ID NO:4 is 35,049 daltons and closely
approximates the molecular weight of P37 of 35-37 kDa as
estimated by apparent mobility in SDS-PAGE.
35   Most preferably, the preparation would have at least 90%
the activity of the PL scramblase preparation described below.
        In one embodiment of the invention, the PL scramblase is
isolated from erythrocyte membranes. In another embodiment,

the protein is produced by bacteria cells, such as *E. coli*
cells, insect cells, or yeast, preferably *in vitro* cultures
that are transfected with plasmid or viral vectors containing
cDNA sequences identified at SEQ ID NO:2 in the correct

5   reading frame (3) or cDNA sequences identified at SEQ ID NO:3
in correct reading frame (1). The vector can be chosen from
among protein expression vectors known to those skilled in the
art. Preferable viral vectors include retrovirus, adenovirus,
and baculovirus vectors.

10   The present invention is also an inhibitor of the PL
scramblase activity of P37. In one embodiment, the inhibitor
is an antisense nucleotide derived from the DNA sequence
encoding P37. One of skill in the art would know how to
create such an antisense nucleotide from the cDNA sequence of

15   P37. In another embodiment, the inhibitor is an antibody,
preferably a monoclonal antibody, raised against P37. One of
skill in the art would know how to make an antibody
preparation from the purified protein preparation described
below. For example, one could isolate the purified P37

20   protein from the electrophoretic gel band and use this
preparation to inject into an antibody-producing host, such as
a rabbit, sheep, goat, rat, or mouse. In addition to
polyclonal immune serum, the antibody-producing splenic
lymphocytes from such animals can be obtained to prepare

25   monoclonal antibody reactive with specific peptide segments of
P37. Such methods are well-known to those skilled in the art
and include myeloma fusion and the *in vitro* culture, cloning
and selection of the resulting hybridoma cells to obtain
monoclonal IgG. In a preferred embodiment, recombinant

30   antibody fragments consisting of antigen-binding Fv or ligated
single chain Ig ScFv can be prepared from a cDNA library
derived from the polyA mRNA of the immune splenic lymphocytes.
In this embodiment, PCR is used to selectively amplify cDNA
encoding the CDR3 hypervariable domains of the heavy and light

35   Ig chains, this cDNA is then ligated into appropriate phage
expression vectors, and the recombined or fused single chain
Fv are then expressed as soluble protein and on the surface of
the phage particles that are propagated in E. Coli or other

suitable host cell. The resulting express recombined
monoclonal Ig Fv on their surface and can be panned and cloned
to isolate unique cDNA encoding for specific monoclonal
reagents that bind to select epitopes on P37 antigen. This
5   unique cDNA can then amplified and used to express large
quantities of the desired monoclonal antibody reagent in an
appropriate expression system. In another embodiment, the
anti-P37 Fv reagent is humanized to reduce its antigenicity
for purposes of injection into a human host. The humanized Fv
10   is prepared by converting the nucleotide segments of the
murine-derived cDNA to the homologous human sequence in those
segments of the heavy and light chain Ig polypeptides that are
flanking on either side of the actual antigen combining site.
The methods for preparation of these various recombinant
15   immunoglobulin reagents are well-known to those skilled in the
art and are also commercially available in kit form (e.g.
Pharamacia ScFv).

In another embodiment, the inhibitor is a peptide derived
from P37. This peptide sequence is an antagonist or
20   competitive inhibitor of P37 PL scramblase activity. The
inhibitor activity could take the form of competing with the
binding of native P37 with plasma membrane phosphatidylserine
or competing with native P37 in calcium binding. One of skill
in the art would realize that one could examine different
25   peptide fragments of P37 for suitability as a competitive
inhibitor.

The present invention is also a method for preventing the
surface exposure of plasma membrane phospholipids, such as
phosphatidylserine, phosphatidylethanolamine and cardiolipin,
30   on the surface of *in vitro* stored platelets or red blood cells
by adding an inhibitor of the PL scramblase activity of P37 to
the stored platelets or red blood cells.

The present invention is also a method for prolonging
survival of transplanted organs and grafts comprising the step
35   of adding an inhibitor of P37 PL scramblase activity to an
organ perfusate during *in vitro* organ storage. The present
invention is also a method for prolonging the survival of
transplanted cells, tissues, and organs by genetically

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engineering the cells to be transplanted so as to alter their
expression of plasma membrane P37 in order to reduce exposure
of PS and other thrombogenic phospholipids at the plasma
membrane surface, thereby reducing the risk of infarction due
5   to fibrin clot formation.

Therefore, in one embodiment, the present invention is a
genetically engineered cell for transplantation into a human
or animal wherein the cell has a lowered PL scramblase
expression. Preferably, the cell expresses no PL scramblase.
10   Preferably, this cell comprises a nucleotide molecule which is
expressed by the cell and which codes for protein inhibiting
the activity of PL scramblase. In another preferable
embodiment, the promotor of the PL scramblase gene is altered
to either increase or decrease the expression of the gene.
15   One of skill in the art of molecular biology would envision
methods to create these altered cells.

Preferably, the engineered cell is selected from the
group consisting of endothelial cells, fibroblasts, epithelial
cells, skeletal cells, cardiac and smooth muscle cells,
20   hepatocytes, pancreatic islet cells, bone marrow cells,
astrocytes, and Schwann cells. The present invention is also
a prosthesis for implantation in an animal or human having the
genetically engineered cells attached thereto. In one
embodiment, the prosthesis is a vascular graft.
25   The present invention is also a method for prolonging the
in vivo survival of circulating blood cells comprising the
step of preventing surface exposure of plasma membrane
phosphatidylserine on the circulating blood cells plasma
membrane P37. One may also wish to prevent the procoagulant
30   properties of erythrocytes in sickle cell disease by
inhibiting erythrocyte P37 in a sickle cell patient.

The present invention is also a method for treating
autoimmune and inflammatory diseases, such as disseminated
intravascular coagulation, vascular thrombosis, fibrin
35   generation during cardiopulmonary bypass procedures,
rheumatoid arthritis, systemic lupus erythematosus, thrombotic
thrombocytopenic purpura, heparin-associated thrombosis, and

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organ transplant rejection comprising the step of treating a
patient with an inhibitor of the PL scramblase activity P37.

The present invention is also a method for diagnosing
individuals with reduced or elevated capacity for
5   platelet-promoted or erythrocyte-promoted fibrin clot activity
by quantitating the level of cellular expression of P37 in the
individual. This method may be performed by using an antibody
to P37 in an immunoblot or ELISA method. The method may also
be performed using oligonucleotides derived by P37 cDNA in the
10 polymerase chain reaction. In another embodiment, the method
may be performed by isolating P37 from a whole blood sample,
measuring the amount of P37 isolated and comparing the
measurement with a control sample.

One may wish to use the protein preparation of the
15 present invention as a hemostatic agent by topically applying
the protein preparation to a wound area in a freely bleeding
patient.

EXAMPLES

A.   Experimental Procedures.

20   **Abbreviations used.   PL,** phospholipid(s); **PC,**
phosphatidylcholine; **PS,** phosphatidylserine; **PE,**
phosphatidylethanolamine; **NBD-PC,**
1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-
sn-glycero-3-phosphocholine; **NBD-PS,**
25 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-
sn-glycero-3-phosphoserine; **OG,** N-Octyl-β-D-glucopyranoside;
PIP$_2$, phosphatidylinositol 4,5-bisphosphate; **APLTase,**
aminophospholipid translocase; **IOVs,** inside-out vesicles.

Materials.   Egg yolk phosphatidylcholine (PC), brain
30 phosphatidylserine (PS), 1-palmitoyl 2-oleoyl phosphatidic
acid,
1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-
sn-glycero-3-phosphocholine (NBD-PC) and
1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-
35 sn-glycero-3-phosphoserine (NBD-PS) were obtained from Avanti
Polar Lipids. N-Octyl-β-D-glucopyranoside (OG) was purchased

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from Calbiochem. Sodium dithionite ($Na_2S_2O_4$, Sigma) was
freshly dissolved in 1 M Tris pH 10 at a concentration of 1 M.

PL Scramblase isolation. Human erythrocytes were
obtained from The Blood Center of Southeastern Wisconsin.
5   Washed erythrocytes were lysed in 10 vol of ice cold 5 mM
sodium phosphate, 1 mM EDTA, pH 7.4, washed free of
hemoglobin, and solubilized at a final protein concentration
of 3.5 mg/ml in the presence of 100 mM OG in buffer A (20 mM
Tris, 0.1 mM EGTA, pH 7.4). After incubation for 30 minutes
10   at 4°C with continuous stirring, samples were centrifuged at
100,000 x g for 30 minutes. The cytoskeleton-depleted
supernatants were loaded on a Poros 20 HQ anion-exchange
column (PerSeptive Biosystems) equilibrated with buffer B (20
mM Tris, 0.1 mM EGTA, 50 mM OG, pH 7.4). Elution was
15   performed with a gradient of 0-400 mM NaCl in buffer B.
Active fractions were pooled, subjected to a buffer exchange
by PD 10 gel filtration (Pharmacia) into buffer C (50 mM
acetate, 0.1 mM EGTA, 50 mM OG, pH 5.0), and loaded on a Poros
20 HS cation-exchange column (PerSeptive Biosystems)
20   equilibrated in buffer C. The breakthrough fraction was
passed through a PD 10 column equilibrated in buffer D (25 mM
PIPES, 1 mM $CaCl_2$, 50 mM OG, pH 6.0) and loaded on a Reactive
Red 120 6% agarose column (Sigma). Elution was performed with
a gradient of 0-1.5 M NaCl in buffer D. Active fractions were
25   pooled and further purified on a Bio-Sil SEC-250 size
exclusion column (Bio-Rad) equilibrated with buffer D in which
$CaCl_2$ was replaced by 0.1 mM EGTA. Due to losses encountered
through size exclusion chromatography, enriched fractions of
PL scramblase were routinely prepared by replacing this step
30   by a second anion exchange fractionation on Mono Q
(Pharmacia). All column chromatography procedures were
performed at room temperature on a BioCAD 20 perfusion
chromatography workstation (PerSeptive Biosystems).

Reconstitution into proteoliposomes. A mixture of PC and
35   PS (9:1 molar ratio) was dried under a stream of nitrogen and
resuspended in buffer E (100 mM Tris, 100 mM KCl, 0.1 mM EGTA,

pH 7.4) by vigorous vortexing. Protein samples were mixed
with liposomes at a final lipid concentration of 4 mg/ml in
the presence of 60 mM OG. Detergent was removed by dialysis
overnight at 4°C against 200 vol of buffer E containing 1 g/L
5   SM2-Biobeads (BioRad).

       Labeling of proteoliposomes with NBD-PL. Proteoliposomes
were labeled with fluorescent NBD-PC or NBD-PS (0.25 mole%)
selectively incorporated into outer, inner, or both membrane
leaflets (E. M. Bevers, *et al., supra*, 1995; J. C. McIntyre
10   and R. G. Sleight, *Biochemistry* 30:11819-11827, 1991). Outer
leaflet-labeled proteoliposomes were prepared by addition of
NBD-PL (in DMSO) to the pre-formed proteoliposome suspension.
Symmetrically-labeled proteoliposomes were prepared by adding
NBD-PL to the PC-PS mixture in $CHCl_3$, before drying lipids
15   under nitrogen (*above*). Addition of protein sample in OG and
dialysis against buffer E was then performed as above.
Proteoliposomes with fluorescent NBD-PL located exclusively in
the inner leaflet of the bilayer were obtained by incubating
symmetrically-labeled proteoliposomes with 20 mM dithionite
20   for 1 minute, in order to chemically and irreversibly quench
fluorescent analogs located in the outer leaflet (J. C.
McIntyre and R. G. Sleight, *Biochemistry* 30:11819-11827,
1991). Vesicles were gel filtered in buffer E to remove
unincorporated material and used immediately for assay of PL
25   scramblase activity.

       PL Scramblase activity. PL scramblase activity was
measured using a modification of an assay previously described
(E. M. Bevers, *et al., supra*, 1995; J. C. McIntyre and R. G.
Sleight, *supra*, 1991). Routinely, NBD-PL outside-labeled
30   proteoliposomes (0.4 mg/ml PL final concentration) were
incubated at 37°C in buffer E in presence or absence of 2 mM
$CaCl_2$. At times indicated in figure legends, proteoliposomes
were diluted 25-fold in buffer E containing 4 mM EGTA, and
transferred to a stirred fluorescence cuvet at 23°C. Initial
35   fluorescence was recorded (SLM Aminco 8000 spectrofluorimeter;
excitation at 470 nm, emission at 532 nm), and 20 mM

dithionite added with fluorescence continuously monitored for
total of 90 seconds. The difference in residual
(non-quenchable) fluorescence observed for samples
preincubated at 37°C in presence vs. absence of 2 mM $CaCl_2$ was

5   attributed to Ca^{2+}-induced change in NBD-PL located in the
outer leaflet, and hence accessible to dithionite (J. C.
McIntyre and R. G. Sleight, supra, 1991). Maximum (100%)
dithionite quenching was determined by addition of 1% Triton
X-100. In all cases, NBD fluorescence was corrected for

10   photobleaching (<6%; measured in absence of dithionite).
Ionized [Ca^{2+}] (Fig. 4) was calculated using FreeCal version
4.0 software (generously provided by Dr. Lawrence F. Brass,
University of Pennsylvania, Philadelphia, PA).

       Trypsination. Samples containing active PL scramblase

15   were incubated for 3 hours at 37°C in the presence of 2 µg/ml
trypsin in 100 mM Tris, 0.1 mM EGTA, pH 8.4, and the reaction
was stopped by addition of 1 mM diisopropylfluorophosphate.
Samples incubated in absence of trypsin or in presence of
trypsin plus 4 µg/ml soybean trypsin inhibitor served as

20   controls. Residual PL scramblase activity of each sample was
then determined following reconstitution into NBD-PS
outside-labeled liposomes. Residual PL scramblase detected in
trypsin-treated samples was normalized to that observed for
identically matched samples incubated without trypsin.

25   **Preparation of erythrocyte IOVs.** Erythrocyte inside out
vesicles (IOVs) were prepared essentially by methods described
by Steck and Kant (T. L. Steck and J. A. Kant, Methods
Enzymol. 31a:172-180, 1974) with minor modifications. Assays
for acetylcholinesterase (outside) and glyceraldehyde

30   3-phosphate dehydrogenase accessibility (inside) (T. L. Steck
and J. A. Kant, supra, 1974) confirmed that >90% of these IOVs
were properly oriented and resealed.

       PL Scramblase activity in IOVs. IOVs were suspended in
10 mM Tris, 140 mM KCl, 7.5 mM NaCl, 0.1 mM EGTA, pH 7.4 at

35   100 µg protein/ml. NBD-PLs (PC or PS) were added to a final

0.25 mole% of total PL. After incorporation of label, IOVs
were incubated in presence 0-2 mM CaCl$_2$ for 15 minutes at
37°C. For each sample, percentage of NBD-PL displaced from
the external to inward-facing membrane leaflet was determined
5   by the BSA "back-exchange" method as previously described (E.
M. Bevers, *et al., supra*, 1995). PL scramblase activity was
evaluated by comparing the amount of NBD-PL moved to the
inward-facing leaflet of IOVs in the presence of Ca^{2+} to that
observed in the presence of 0.1 mM EGTA. Transport of
10 dithionite by erythrocyte membrane anion exchanger precludes
use of this quencher to monitor NBD-PL distribution in IOVs
(T. Pomorski, *et al., Mol. Membr. Biol.* 11:39-44, 1994).

B.   Results and Discussion
        Purification and membrane reconstitution of erythrocyte
15 **PL scramblase.** Human erythrocyte membrane proteins depleted
of cytoskeleton were solubilized with OG and initially
fractionated by anion exchange chromatography. Each eluting
fraction was reconstituted into PL vesicles and then 0.25
mole% NBD-PS added to the outer leaflet. After incubation in
20 the presence of either 0 or 2 mM Ca^{2+}, the time-dependent
distribution of NBD-PS between inner and outer leaflets was
determined (see *Experimental Procedures* and below). A
Ca^{2+}-dependent movement of NBD-PS from outer to inner membrane
leaflets was detected in only those vesicles reconstituted
25 with proteins eluting from Poros 20 HQ between 125 mM-200 mM
NaCl. Such activity was not detected in any other column
fraction, including those enriched with the predominant
erythrocyte membrane band 3 protein, nor was this activity
found in unbound flow-through fractions (not shown). Of note,
30 erythrocyte band 3 protein has been reported to exhibit
intrinsic "flipping activity" for anionic PL, based on the
capacity of the anion transport inhibitor
4,4'-diisothiocyanostilbene-2,2'-disulfonic acid to further
attenuate the slow spontaneous (Ca^{2+}-*independent*) migration PL
35 across the erythrocyte membrane (A. Vondenhof, *et al.,*
Biochemistry 33:4517-4520, 1994).

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Through subsequent purification, this PL scramblase
activity was found to co-purify with a ~37 kDa protein band
that we were able to enrich to apparent homogeneity (Fig. 1).
Fig. 1 illustrates final stage of purification of erythrocyte
5   PL scramblase activity. Pool of activity eluting from
Reactive Red 120 was concentrated and applied to a 600 x 7.5
mm BioSil SEC250 column. Flow rate was 0.2 ml/min and 1.0 ml
fractions were collected. 100 μl aliquots of each column
fraction were reconstituted into liposomes containing NBD-PS
10 exclusively in the outer leaflet and assayed for PL scramblase
activity. Samples were also concentrated 10-fold and analyzed
by 12.5% SDS-PAGE under reducing conditions, developed with
silver staining. Relative PL scramblase activity is plotted
directly above the corresponding gel lane. Only data for
15 fractions eluting about peak of activity (fraction #12) are
shown. See Experimental Procedures. Fractions 11 and 13
contained additional bands not represented in Fig. 1B.

The activity of this PL scramblase was destroyed by
trypsin (Table 1), confirming that activity detected in the
20 reconstituted proteoliposome derived from the purified protein
fraction, and not other potentially co-eluting material. In
this context, PL scramblase activity has recently been
attributed to an interaction of Ca^{2+} directly with membrane
lipid. As noted above (*Introduction*), Sulpice, *et al.* (J. C.
25 Sulpice, *et al.*, *supra*, 1994) concluded that Ca^{2+}-binding to
PIP_2 located in the inner leaflet of the erythrocyte membrane
induces transbilayer PL scrambling, although this conclusion
was not supported by a subsequent study (E. M. Bevers, *et al.*,
supra, 1995). Alternatively, Bratton proposed that
30 cytoplasmic polyamines contribute to maintenance of PL
asymmetry by shielding anionic head groups from Ca^{2+}, and that
enhanced transbilayer movement of PL might arise through loss
of polyamine-membrane associations at increased intracellular
Ca^{2+} (D. L. Bratton, *supra*, 1994). Although our studies do
35 not address a potential influence of these cytoplasmic
components on PL scramblase activity, it should be noted that
polyamines would be removed through our lysis and
solubilization procedures. Finally, sensitivity of PL

scramblase to inactivation by trypsin (Table 1) excludes the
possibility that the activity we observe arises from
contaminating PIP_2 or polyamine. Although data of Fig. 1 and
Table 1 suggest that PL scramblase is a low abundance integral

5   membrane polypeptide of ~37 kDa, we cannot now exclude the
possibility that observed activity resides in a trace
co-purifying membrane protein that goes undetected by silver
staining.

Characteristics of PL transport mediated by isolated PL

10   **scramblase.** Proteoliposomes reconstituted with purified PL
scramblase remained impermeant to dithionite and stable to
spontaneous NBD-PS exchange in absence of Ca^{2+} (Fig. 2).

Fig. 2 illustrates bidirectional movement of NBD-PS
across lipid bilayers reconstituted with PL scramblase. PL

15   scramblase was reconstituted into 90% PC, 10% PS membranes
trace-labeled with 0.25% NBD-PS, initially located either in
the inner membrane leaflet (inside-labeled), randomly
distributed between leaflets (symmetrically-labeled), or
exclusively in the outer leaflet (outside-labeled). Vesicles

20   were incubated 3 hours at 37°C in presence of either 0.1 mM
EGTA (dotted lines) or 2 mM Ca^{2+} (solid lines) and reaction
stopped with EGTA. Distribution of NBD-PS in outer leaflet
was determined by quenching with 20 mM dithionite, time of
addition indicated by arrow (see Experimental Procedures).

25   Ordinate denotes NBD fluorescence, normalized to t=0; abscissa
denotes fluorescence acquisition time (sec). Dashed line
indicates level of fluorescence detected after addition of 1%
Triton X-100 to each vesicle suspension in presence of
dithionite (100% quenching). In this experiment, observed

30   Ca^{2+}-dependent transbilayer movement of NBD-PS equaled 8±2%
(inside labeled), 0±1% (symmetrically-labeled), and 8±1%
(outside-labeled). Fig. 2 illustrates the data of single
experiment, representative of at least three separate
experiments so performed.

35   As also illustrated by Fig. 2, Ca^{2+}-induced PL scramblase
activity was observed to be bidirectional, induced
sequestration of NBD-PS initially distributed only in the

outer membrane leaflet equaling induced exposure of NBD-PS
initially distributed only in the inner leaflet. In
proteoliposomes prepared with NBD-PS randomly distributed
between membrane leaflets, no movement of probe was detected

5   upon Ca^{2+} addition, consistent with one-for-one PL exchange
between membrane leaflets. Similar results were obtained when
NBD-PC substituted for NBD-PS, and movement of either
NBD-labeled PL was unaffected by replacement of matrix PS by
phosphatidic acid (data not shown). These results imply that

10 the PL scramblase activity exhibited by lipid vesicles
reconstituted with this 37 kDa protein is non-selective for PS
versus PC, and facilitates both inward and outward movement of
PL between membrane leaflets. Assuming that movement of PL
initiated by addition of Ca^{2+} arises through activation of

15 only those proteins oriented to expose Ca^{2+}-binding site(s) at
the external face of the liposome, these data imply that each
PL scramblase mediates bidirectional and non-selective
one-for-one exchange of PL between membrane leaflets.
Although it has been suggested that elevation of cytosolic

20 Ca^{2+} initiates a vectorial movement of PS and PE from inner to
outer plasma membrane leaflets (Bassé, et al., supra, 1993; P.
Gaffet, et al., supra, 1995), more recent data suggest that
this apparent egress of inner leaflet PS arises through random
bidirectional exchange of PL residing in both leaflets of the

25 plasma membrane (P. Williamson, et al., supra, 1992; E. F.
Smeets, et al., supra, 1994; P. Williamson, et al., supra,
1995), consistent with the activity we observe in PL
scramblase reconstituted proteoliposomes. The fact that these
proteoliposomes remain impermeant to dithionite (see Fig. 2),

30 excludes the possibility that this apparently random and
bidirectional movement of lipid arises through lytic
disruption of lipid bilayer. Nevertheless, we cannot exclude
the possibility that an initially vectorial transport of PL in
these small vesicles is masked by a counterbalancing exchange

35 to maintain mass balance between membrane leaflets, as might
be imposed by head group packing constraints.
        The mechanism by which Ca^{2+} interaction with this protein
initiates transbilayer movement of PL remains unresolved. To

be noted, we observed only a requirement for Ca^{2+} with no requirement for either Mg^{2+} or ATP. In presence of 2 mM Ca^{2+}, lipid movement catalyzed by PL scramblase obeyed pseudo-first order kinetics with initial rates that increased with
5   concentration of protein, consistent with a facilitated transport or carrier mechanism (Fig. 3).

Fig. 3 diagrams the kinetics of PS translocation by reconstituted PL scramblase. Proteoliposomes were prepared with a constant amount of PL and 2-fold dilutions of purified
10   PL scramblase (*Experimental Procedures*). NBD-PS (0.25 mole%) was added to outer leaflet and labeled proteoliposomes incubated at 37°C in presence of 2 mM Ca^{2+}. At times indicated (*abscissa*) samples were analyzed for the amount of NBD-PS that migrated from outer to inner leaflets, according
15   to assay shown in Fig. 2. All data are corrected for spontaneous transbilayer migration of NBD-PS ($\leq 1\%$ hr^{-1}) measured for identical samples incubated with 0.1 mM EGTA, substituting for Ca^{2+}. Fig. 3 illustrates the data at highest protein concentration (O); 2-fold (■); 4-fold (●) and 8-fold
20   dilutions (□) are indicated. Experimental points at each protein concentration were fitted to first-order exponential rate equation (Sigma Plot; *dotted lines*). *Insert:* Derived initial rates (V_i; *ordinate*) plotted against normalized protein concentration (*abscissa*). Data of single experiment,
25   representative of two so performed.

Derived rate constants for transbilayer lipid movement (at 37°C) ranged between 0.45 - 0.74 hr^{-1} over an eight-fold range of protein/lipid ratio. Whereas this activity deviated from anticipated linearity with increased added protein per
30   lipid (insert, Fig. 3), this may reflect an inherent limitation in efficiency of protein reconstitution into the liposome membrane (*below*). The apparent rate of Ca^{2+}-evoked transbilayer movement attained in the reconstituted system ($t_{\frac{1}{2}}$ -2 hr; Fig. 3) is approximately one-fourth of the observed
35   rate of PL scrambling ($t_{\frac{1}{2}}$ -0.5 hr; P. Williamson, *et al.*, *supra*, 1992) induced in Ca^{2+}-ionophore treated erythrocytes, suggesting somewhat reduced activity in the reconstituted system. Whether this reduced activity reflects partial

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denaturation of PL scramblase, or, our inability to incorporate amounts of this protein equivalent to that present in the erythrocyte membrane, remains to be determined.

Calcium dependence. In order to confirm that the
5 activity of reconstituted PL scramblase mimicked intrinsic PL scramblase activity of the erythrocyte membrane, we compared $[Ca^{2+}]$-dependence of PL movement in proteoliposomes reconstituted with purified protein to that observed in erythrocyte IOVs (Fig. 4). Fig. 4 diagrams Ca^{2+} dependence of
10 PL scramblase activity in proteoliposomes & erythrocyte IOVs. PL scramblase activity of reconstituted proteoliposomes (*closed symbols*) and erythrocyte IOVs (*open symbols*) was determined as described in *Experimental Procedures*, and plotted as function of external free $[Ca^{2+}]$ (●,O). Data for
15 each sample were normalized to maximum PL scramblase activity, measured at 2 mM Ca^{2+}. *Squares* (■,□) denote PL scramblase activity detected when Mg^{2+} substituted for Ca^{2+}. Data of single experiment, representative of three so performed.

In both IOVs and isolated protein reconstituted
20 liposomes, PL scramblase activity was found to obey a sigmoidal dose-response to Ca^{2+}, with saturation at ~100 μM $[Ca^{2+}]$, and half-maximal activities attained between 20-60 μM Ca^{2+}. In both cases, little effect of Mg^{2+} was observed. These results are similar to data previously reported for
25 Ca^{2+}-induced movement of plasma membrane PL in ionophore-treated erythrocytes, resealed erythrocyte ghosts, and other cells (H. Kojima, et al., *Biochemistry* 33:4517-4520, 1994; B. Verhoven, et al., *Biochim. Biophys. Acta* 1104:15-23, 1992).
30 PL scramblase activity found in erythrocyte membranes is also observed in platelets, lymphocytes and other cells and is thought to play a central role in initiation of fibrin clot formation and in recognition of apoptotic and injured cells by the reticuloendothelial system. It is not known whether the
35 same protein is responsible for PL scramblase activity found in other cells, or whether such proteins are cell-specific. A protein common to all blood cells is suggested by the

observation that the genetic defect in PL scramblase activity
arising in Scott syndrome affects all hematologic lineages.
Further insight into the cellular distribution of the 37 kDa
protein that we have now isolated from normal erythrocytes
5   awaits purification of sufficient protein for sequencing, and
to develop specific antibodies.

Table 1. Inactivation of PL scramblase by trypsin

Treatment		PL scramblase activity[a] (% of control)
Trypsin	Soybean Trypsin Inhibitor	
-	-	(100)
-	+	100
+	-	13
+	+	100

[a]PL scramblase in response to 2 mM Ca^{2+}, expressed as percent
15   of activity of untreated control. Incubation with trypsin and
reconstitution of digested protein into liposomes for PL
scramblase assay are detailed under *Experimental Procedures*.
Range of measured activities ± 5%. Representative of two
separate experiments.

20   **C.   Amino Acid and Nucleotide Sequence of the PL Scramblase.**
We purified the PL scramblase by the methods described above.
The resulting protein was subjected to further purification by
sodium dodecyl sulfate polyacrylamide gel electrophoresis, the
protein band visualized by staining with Coomassie brilliant
25   blue, and a slice of the wet gel containing the P37 protein
band migrating between ~35-37 kDa excised. This gel slice was
sent to the Protein and Carbohydrate Structure Facility at
University of Michigan, Ann Arbor, Michigan for cyanogen
bromide digestion and internal peptide sequencing. Peptide
30   sequence was obtained from a 2 kD cyanogen bromide fragment
derived from the protein contained in the gel slice. The
amino acid sequence we obtained is disclosed in residues 59 -
90 of SEQ ID NO:1.
     We obtained an exact match for these residues with a
35   translation product of a cDNA reported in Genbank at AA14325.

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This sequence includes the predicted methionine (residue 58 of
SEQ ID NO:1) located 5' to the preferred locus of cyanogen
bromide peptide bond cleavage, yielding a total of 33 residues
of continuous identity between the deduced partial internal
5   sequence from P37 and the translation product of the cDNA
clone reported in Genbank at AA143025. The entire nucleotide
sequence of this cDNA clone (ATCC 962235) is reported at SEQ
ID NO:2. Sequence reported at SEQ ID NO:1 is the entire
translated sequence of SEQ ID NO:2, using the correct
10 translation reading frame that we deduced from comparison of
the cDNA clone with residues 58 - 90 of SEQ ID NO:1.

Using methods standard to those skilled in the art, we
probe human leukocyte, B-lymphocyte, fetal adrenal, and K562
(a human erythro-leukemic cell line) cDNA libraries by PCR,
15 using oligonucleotide primers designed to anneal specifically
to SEQ ID NO:2. The PCR reactions revealed that cDNA
overlapping SEQ ID NO:2 of the expected number of residues was
contained in each of these cDNA libraries. This result
indicates that mRNA encoding sequence contained in P37 protein
20 purified from human erythrocyte membrane is also expressed in
multiple human cell lines of both erythroid and non-erythroid
origin. Based on evidence for the relative abundance of cDNA
overlapping SEQ ID NO:2 in the Clontech lambda gt11 K562
library, we selected this library to amplify additional cDNA
25 sequence flanking SEQ ID NO:2.

Using methods standard to those skilled in the art, we
PCR amplified the flanking DNA sequences 5' and 3' to sequence
ID NO:2 from the K562 lambda gt11 cDNA library (Clontech, Inc)
with oligonucleotide primers designed to anneal to sequence ID
30 NO:2 in combination with oligonucleotide primers designed to
anneal to regions of the Clontech lambda gt11 vector in the
vicinity of the multiple cloning site of the vector. The
resulting PCR-amplified cDNA was purified and sequenced. From
these PCR-amplified 5' and 3' flanking sequences, partial
35 overlapping identities were found in cDNA reported in Genbank
at AA143025, AA056199, AA054476, AA151006, AA171663, D61890,
H42131, R76171, R66275, R82537, W90481 and these multiple
sequences used in combination with direct sequence obtained

from our PCR products to deduce and to confirm the sequence
reported at SEQ ID NO:3.

SEQ ID NO:4 is the entire translated sequence of SEQ ID
NO:3, using the correct translation reading frame that we
5   deduced from comparison of the cDNA clone with peptide
residues 58 - 90 of SEQ ID NO:1.

The open reading frame of the sequence reported at SEQ ID
NO:3 predicts a polypeptide of 318 amino acids (identified as
residues **Met75- Trp392** of SEQ ID NO:4) with the calculated
10   molecular weight of 35,049 and theoretical isolectric point
(pI) = 4.83. This closely approximates the experimentally
estimated molecular weight and isolectric point of P37 PL
scramblase purified from erythrocyte membranes. Thus we
identify residues 75 - 392 of SEQ ID NO:4 as P37, human plasma
15   membrane PL scramblase and deduce that this same protein is
expressed in both erythroid and non-erythroid human cells.

Analysis of the sequence contained in P37 (identified as
residues 75 - 392 of SEQ ID NO:4) predicts a membrane protein
with a cytoplasmic N-terminus and a single transmembrane helix
20   formed by residues 365 - 383, with residues 384 - 392
projecting from the cell surface and residues 75 - 364
internal to the cell. A site for phosphorylation by protein
kinase C or other serine/threonine protein kinase is predicted
at P37 Thr residue 235 (all residue numbers for P37 given
25   according to sequence identified at SEQ ID NO:4). It has been
observed that activity of PL scramblase is reduced in cells
depleted of ATP (Martin, et al., J. Biol. Chem. 270:10468-
10474, 1995), and that inhibitors of Ca^{2+}/calmodulin kinases
reduce platelet plasma membrane microparticle formation
30   (Wiedmer, et al., Blood 78:2880-2886, 1991), which is
consistent with the regulation of the activity of PL
scramblase by a phosphorylation of P37 mediated by one or
more intracellular protein kinases.

SEQUENCE LISTING

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    (ii) TITLE OF INVENTION: A PROTEIN PREPARATION THAT MEDIATES
                                Ca^{2+}-DEPENDENT TRANSBILAYER MOVEMENT OF PLASMA
                                MEMBRANE PHOSPHOLIPID AND INHIBITORS THEREOF

    (iii) NUMBER OF SEQUENCES: 4

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    (v) COMPUTER READABLE FORM:
        (A) MEDIUM TYPE: Floppy disk
        (B) COMPUTER: IBM PC compatible
        (C) OPERATING SYSTEM: PC-DOS/MS-DOS
        (D) SOFTWARE: PatentIn Release #1.0, Version #1.25

    (vi) CURRENT APPLICATION DATA:
        (A) APPLICATION NUMBER:
        (B) FILING DATE:
        (C) CLASSIFICATION:

    (viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 188 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

    Ala Phe Gln Gly Pro Pro Gly Tyr Ser Gly Tyr Pro Gly Pro Gln Val
    1   5   10   15

    Ser Tyr Pro Pro Pro Ala Gly His Ser Gly Pro Gly Pro Ala Gly
             20   25   30

    Phe Pro Val Pro Asn Gln Pro Val Tyr Asn Gln Pro Val Tyr Asn Gln
             35   40   45

```
Pro Val Gly Ala Ala Gly Val Pro Trp Met Pro Ala Pro Gln Pro Pro
    50                  55              60

Leu Asn Cys Pro Pro Gly Leu Glu Tyr Leu Ser Gln Ile Asp Gln Ile
65               70              75              80

Leu Ile His Gln Gln Ile Glu Leu Leu Glu Val Leu Thr Gly Phe Glu
            85              90              95

Thr Asn Asn Lys Tyr Glu Ile Lys Asn Ser Phe Gly Gln Arg Val Tyr
            100             105             110

Phe Ala Ala Glu Asp Thr Asp Cys Cys Thr Arg Asn Cys Cys Gly Pro
        115             120             125

Ser Arg Pro Phe Thr Leu Arg Ile Ile Asp Asn Met Gly Gln Glu Val
    130             135             140

Ile Thr Leu Glu Arg Pro Leu Arg Cys Ser Ser Cys Cys Cys Pro Cys
145             150             155             160

Cys Leu Gln Glu Ile Glu Ile Gln Ala Pro Pro Gly Val Pro Ile Gly
            165             170             175

Tyr Val Ile Gln Thr Trp His Pro Cys Leu Pro Lys
            180             185
```

(2) INFORMATION FOR SEQ ID NO:2:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 568 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: cDNA

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
CAGCATTCCA AGGACCTCCA GGATATAGTG GCTACCCTGG GCCCCAGGTC AGCTACCCAC      60

CCCCACCAGC CGGCCATTCA GGTCCTGGCC CAGCTGGCTT TCCTGTCCCA AATCAGCCAG     120

TGTATAATCA GCCAGTATAT AATCAGCCAG TTGGAGCTGC AGGGGTACCA TGGATGCCAG     180

CGCCACAGCC TCCATTAAAC TGTCCACCTG GATTAGAATA TTTAAGTCAG ATAGATCAGA     240

TACTGATTCA TCAGCAAATT GAACTTCTGG AAGTTTTAAC AGGTTTTGAA ACTAATAACA     300

AATATGAAAT TAAGAACAGC TTTGGACAGA GGGTTTACTT TGCAGCGGAA GATACTGATT     360

GCTGTACCCG AAATTGCTGT GGGCCATCTA GACCTTTTAC CTTGAGGATT ATTGATAATA     420

TGGGTCAAGA AGTCATAACT CTGGAGAGAC CACTAAGATG TAGCAGCTGT TGTTGTCCCT     480

GCTGCCTTCA GGAGATAGAA ATCCAAGCTC CTCCTGGTGT ACCAATAGGT TATGTTATTC     540

AGACTTGGCA CCCATGTCTA CCAAAGTT                                       568
```

(2) INFORMATION FOR SEQ ID NO:3:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 1266 base pairs
        (B) TYPE: nucleic acid
        (C) STRANDEDNESS: double
        (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
CGTGTTGTGT ACGTAAGATT CAGGAAACGA AACCAGGAGC CGCGGGTGTT GGCGCAAAGG      60

TTACTCCCAG ACCCTTTTCC GGCTGACTTC TGAGAAGGTT GCGCAGCAGC TGTGCCCGAC     120

AGTCTAGAGG CGCAGAAGAG GAAGCCATCG CCTGGCCCCG GCTCTCTGGA CCTTGTCTCG     180

CTCGGGAGCG GAAACAGCGG CAGCCAGAGA ACTGTTTTAA TCATGGACAA ACAAAACTCA     240

CAGATGAATG CTTCTCACCC GGAAACAAAC TTGCCAGTTG GGTATCCTCC TCAGTATCCA     300

CCGACAGCAT TCCAAGGACC TCCAGGATAT AGTGGCTACC CTGGGCCCCA GGTCAGCTAC     360

CCACCCCCAC CAGCCGGCCA TTCAGGTCCT GGCCCAGCTG GCTTTCCTGT CCCAAATCAG     420

CCAGTGTATA ATCAGCCAGT ATATAATCAG CCAGTTGGAG CTGCAGGGGT ACCATGGATG     480

CCAGCGCCAC AGCCTCCATT AAACTGTCCA CCTGGATTAG AATATTTAAG TCAGATAGAT     540

CAGATACTGA TTCATCAGCA AATTGAACTT CTGGAAGTTT TAACAGGTTT TGAAACTAAT     600

AACAAATATG AAATTAAGAA CAGCTTTGGA CAGAGGGTTT ACTTTGCAGC GGAAGATACT     660

GATTGCTGTA CCCGAAATTG CTGTGGGCCA TCTAGACCTT TTACCTTGAG GATTATTGAT     720

AATATGGGTC AAGAAGTCAT AACTCTGGAG AGACCACTAA GATGTAGCAG CTGTTGTTGT     780

CCCTGCTGCC TTCAGGAGAT AGAAATCCAA GCTCCTCCTG GTGTACCAAT AGGTTATGTT     840

ATTCAGACTT GGCACCCATG TCTACCAAAG TTTACAATTC AAAATGAGAA AAGAGAGGAT     900

GTACTAAAAA TAAGTGGTCC ATGTGTTGTG TGCAGCTGTT GTGGAGATGT TGATTTTGAG     960

ATTAAATCTC TTGATGAACA GTGTGTGGTT GGCAAAATTT CCAAGCACTG GACTGGAATT    1020

TTGAGAGAGG CATTTACAGA CGCTGATAAC TTTGGAATCC AGTTCCCTTT AGACCTTGAT    1080

GTTAAAATGA AAGCTGTAAT GATTGGTGCC TGTTTCCTCA TTGACTTCAT GTTTTTTGAA    1140

AGCACTGGCA GCCAGGAACA AAAATCAGGA GTGTGGTAGT GGATTAGTGA AAGTCTCCTC    1200

AGGAAATCTG AAGTCTGTAT ATTGATTGAG ACTATCTAAA CTCATACCTG TATGAATTAA    1260

GCTGTA                                                               1266
```

(2) INFORMATION FOR SEQ ID NO:4:

    (i) SEQUENCE CHARACTERISTICS:
        (A) LENGTH: 422 amino acids
        (B) TYPE: amino acid
        (C) STRANDEDNESS: single
        (D) TOPOLOGY: linear

    (ii) MOLECULE TYPE: peptide

    (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Arg Val Val Tyr Val Arg Phe Arg Lys Arg Asn Gln Glu Pro Arg Val
1               5                  10                  15

Leu Ala Gln Arg Leu Leu Pro Asp Pro Phe Pro Ala Asp Phe Xaa Glu
            20              25                  30

Gly Cys Ala Ala Ala Val Pro Asp Ser Leu Glu Ala Gln Lys Arg Lys
            35              40                  45
```

Pro Ser Pro Gly Pro Gly Ser Leu Asp Leu Val Ser Leu Gly Ser Gly
    50   55   60

Asn Ser Gly Ser Gln Arg Thr Val Leu Ile Met Asp Lys Gln Asn Ser
65   70   75   80

Gln Met Asn Ala Ser His Pro Glu Thr Asn Leu Pro Val Gly Tyr Pro
                85   90   95

Pro Gln Tyr Pro Pro Thr Ala Phe Gln Gly Pro Pro Gly Tyr Ser Gly
            100   105   110

Tyr Pro Gly Pro Gln Val Ser Tyr Pro Pro Pro Ala Gly His Ser
        115   120   125

Gly Pro Gly Pro Ala Gly Phe Pro Val Pro Asn Gln Pro Val Tyr Asn
    130   135   140

Gln Pro Val Tyr Asn Gln Pro Val Gly Ala Ala Gly Val Pro Trp Met
145   150   155   160

Pro Ala Pro Gln Pro Pro Leu Asn Cys Pro Pro Gly Leu Glu Tyr Leu
            165   170   175

Ser Gln Ile Asp Gln Ile Leu Ile His Gln Gln Ile Glu Leu Leu Glu
        180   185   190

Val Leu Thr Gly Phe Glu Thr Asn Asn Lys Tyr Glu Ile Lys Asn Ser
    195   200   205

Phe Gly Gln Arg Val Tyr Phe Ala Ala Glu Asp Thr Asp Cys Cys Thr
    210   215   220

Arg Asn Cys Cys Gly Pro Ser Arg Pro Phe Thr Leu Arg Ile Ile Asp
225   230   235   240

Asn Met Gly Gln Glu Val Ile Thr Leu Glu Arg Pro Leu Arg Cys Ser
            245   250   255

Ser Cys Cys Cys Pro Cys Cys Leu Gln Glu Ile Glu Ile Gln Ala Pro
            260   265   270

Pro Gly Val Pro Ile Gly Tyr Val Ile Gln Thr Trp His Pro Cys Leu
    275   280   285

Pro Lys Phe Thr Ile Gln Asn Glu Lys Arg Glu Asp Val Leu Lys Ile
    290   295   300

Ser Gly Pro Cys Val Val Cys Ser Cys Cys Gly Asp Val Asp Phe Glu
305   310   315   320

Ile Lys Ser Leu Asp Glu Gln Cys Val Val Gly Lys Ile Ser Lys His
            325   330   335

Trp Thr Gly Ile Leu Arg Glu Ala Phe Thr Asp Ala Asp Asn Phe Gly
            340   345   350

Ile Gln Phe Pro Leu Asp Leu Asp Val Lys Met Lys Ala Val Met Ile
    355   360   365

Gly Ala Cys Phe Leu Ile Asp Phe Met Phe Phe Glu Ser Thr Gly Ser
    370   375   380

Gln Glu Gln Lys Ser Gly Val Trp Xaa Trp Ile Ser Glu Ser Leu Leu
385   390   395   400

Arg Lys Ser Glu Val Cys Ile Leu Ile Glu Thr Ile Xaa Thr His Thr
                405   410   415

Cys Met Asn Xaa Ala Val
            420

CLAIMS

We claim:

1.   A preparation of a phospholipid scramblase, wherein the protein is approximately 35-37 kD as measured on a 12.5% SDS-polyacrylamide gel under reducing conditions.

2.   The preparation of claim 1, wherein the phospholipid scramblase comprises residues 58 - 90 of SEQ ID NO:1.

3.   The preparation of claim 1, wherein the phospholipid scramblase comprises residues 1 - 188 of SEQ ID NO:1.

4.   The preparation of claim 1, wherein the phospholipid scramblase comprises residues 75-392 of SEQ ID NO:4.

5.   The preparation of claim 1 wherein the protein is isolated from erythrocyte membranes.

6.   The preparation of claim 1 wherein the protein is produced by cells genetically modified so as to express the protein of claim 1.

7.   The preparation of claim 1 wherein the protein is produced by an organism selected from the group consisting of bacteria, insect cells, or yeast.

8.   A recombinant DNA sequence encoding the protein of claim 1.

9.   The sequence of claim 8, comprising SEQ ID NO:3.

10.   The sequence of claim 8, comprising nucleotides 223-1176 of SEQ ID NO:3.

11.   The sequence of claim 8, comprising SEQ ID NO:2 and wherein the sequence is part of a protein expression vector.

12.   An inhibitor of phospholipid scramblase activity of the protein of claim 1.

13.   The inhibitor of claim 12 wherein the inhibitor is an antisense nucleotide derived from a DNA sequence encoding

5   P37.

14.   The inhibitor of claim 12 wherein the inhibitor is an antisense nucleotide derived from a DNA sequence identified at SEQ ID NO:3.

15.   The inhibitor of claim 12 wherein the inhibitor is a

10   monoclonal antibody against P37.

16.   The inhibitor of claim 12 wherein the inhibitor is a monoclonal antibody against peptide sequence identified as residues 385-392 of SEQ ID NO:4.

17.   The inhibitor of claim 15 wherein the monoclonal

15   antibody is selected from the group consisting of recombinantly produced intact IgG, Ig Fab fragment, recombinant Ig Fv or recombinant single chain Ig Fv fragment ScFv.

18.   The inhibitor of claim 12 wherein the inhibitor is a

20   peptide containing an amino acid sequence derived from P37, wherein the peptide is a competitive inhibitor of P37 phospholipid scramblase activity.

19.   The inhibitor of claim 18 wherein the inhibitor is a peptide that binds to plasma membrane phosphatidylserine.

25   20.   The inhibitor of claim 18 wherein the inhibitor is a peptide that prevents binding of Ca^{2+} to P37.

21.   The inhibitor of claim 18 wherein the inhibitor prevents phosphorylation of P37 by a cellular protein kinase.

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22. The inhibitor of claim 21 containing the peptide
sequence Thr-Leu-Arg or is a peptidomimetic structural
analogue of the tripeptide Thr-Leu-Arg.

23. A method for preventing the surface exposure of
5   plasma membrane phosphatidylserine, phosphatidylethanolamine
and cardiolipin on the surface of *in vitro* stored platelets or
red blood cells comprising the step of adding an inhibitor of
P37 to the stored platelets or red blood cells.

24. The method of claim 23 wherein the inhibitor is a
10   nucleotide sequence.

25. The method of claim 23 wherein the inhibitor is a
monoclonal antibody against P37.

26. The method of claim 23 wherein the inhibitor is a
peptide containing an amino acid sequence derived from P37,
15   wherein the peptide is an antagonist of P37 phospholipid
scramblase activity.

27. The method of claim 26 wherein the inhibitor is a
peptide that binds to plasma membrane phosphatidylserine.

28. The method of claim 26 wherein the inhibitor is a
20   peptide that prevents binding of Ca^{2+} to P37.

29. The method of claim 26 wherein the inhibitor is a
peptide or peptidomimetic peptide analogue that prevents
phosphorylation of P37 by a cellular kinase.

30. A method for prolonging graft survival of
25   transplanted organs and grafts comprising the step of adding
an inhibitor of P37 phospholipid scramblase activity to an
organ perfusate during *in vitro* organ storage.

31. A method for preventing the procoagulant properties
of erythrocytes in sickle cell disease comprising the step of
30   inhibiting erythrocyte P37 in a sickle cell patient.

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32. A method for prolonging the *in vivo* survival of circulating blood cells comprising the step of preventing surface exposure of plasma membrane phosphatidylserine on the circulating blood cells by exposing the blood cells to an
5   inhibitor of plasma membrane P37.

33. A method for treating autoimmune, thrombotic, thromboemolic, and inflammatory diseases comprising the step of treating a patient with an inhibitor of the phospholipid scramblase activity of P37.

10   34. The method of claim 33, wherein the disease is selected from disseminated intravascular coagulation, vascular thrombosis, fibrin generation during cardiopulmonary bypass procedures, rheumatoid arthritis, systemic lupus erythematosus, thrombotic thrombocytopenic purpura, heparin-associated
15   thrombosis, and organ transplant rejection.

35. A method for diagnosing individuals with reduced or elevated capacity for platelet-promoted or erythrocyte-promoted fibrin clot activity comprising the step of quantitating the level of cellular expression of P37 in
20   platelets and erythrocytes in a whole blood sample obtained from the patient mixed with acid-citrate-dextrose, EDTA, heparin, or other suitable anticoagulant.

36. The method of claim 35 wherein quantitation is performed by immunoblotting using an antibody to P37.

25   37. The method of claim 35 wherein quantitation is performed by ELISA assay using an antibody to P37.

38. The method of claim 35 wherein quantitation is performed by fluorescence-activated flow cytometry using an antibody to P37.

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39. The method of claim 38 wherein quantitation is
performed using a monoclonal antibody reactive with the peptide
sequence corresponding to residues 385-392 of SEQ ID NO:4.

5   40. The method of claim 35 wherein quantitation is
performed using oligonucleotides derived from P37 cDNA and the
polymerase chain reaction.

41. The method of claim 35 wherein quantitation is
performed by isolating P37 from a patient venous blood sample,
by first isolating the platelets and erythrocytes by
10 differential centrifugation, and then measuring the amount of
P37 present either per cell or per unit of total cell protein,
and comparing the measurement with control sample.

42. A genetically engineered cell for transplantation
into a human or animal wherein the cell does not express PL
15 scramblase at native levels of expression.

43. The cell of claim 42 further comprising a nucleotide
molecule which is expressed by the cell and which codes for
protein inhibiting the activity of PL scramblase.

44. The cell of claim 42 wherein the promoter of the PL
20 scramblase gene is altered to either increase or decrease the
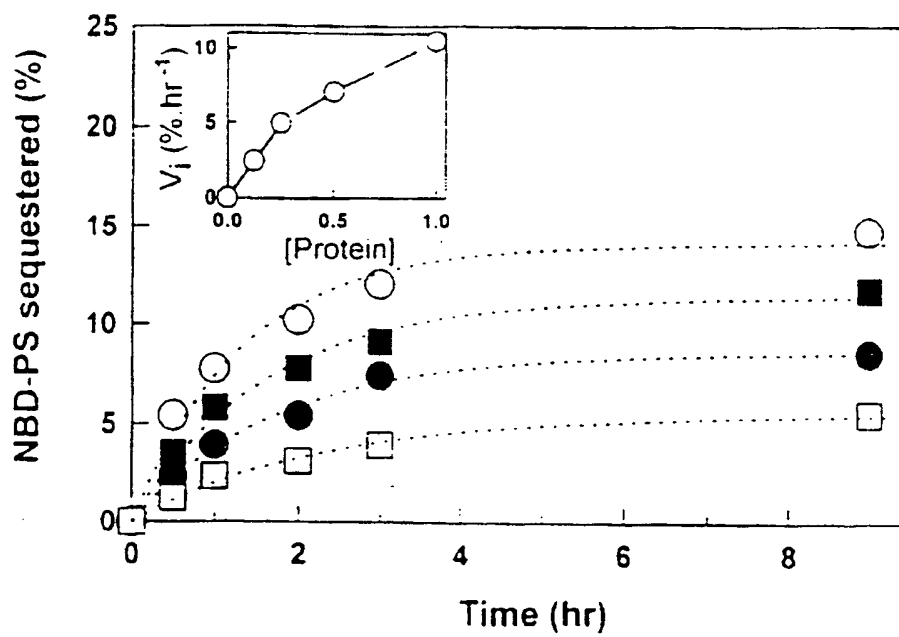expression of the gene.

45. The cell of claim 42 selected from the group
consisting of endothelial cells, fibroblasts, epithelial
cells, skeletal cells, cardiac and smooth muscle cells,
25 hepatocytes, pancreatic islet cells, bone marrow cells,
astrocytes, and Schwann cells

46. A prothesis for implantation in an animal or human
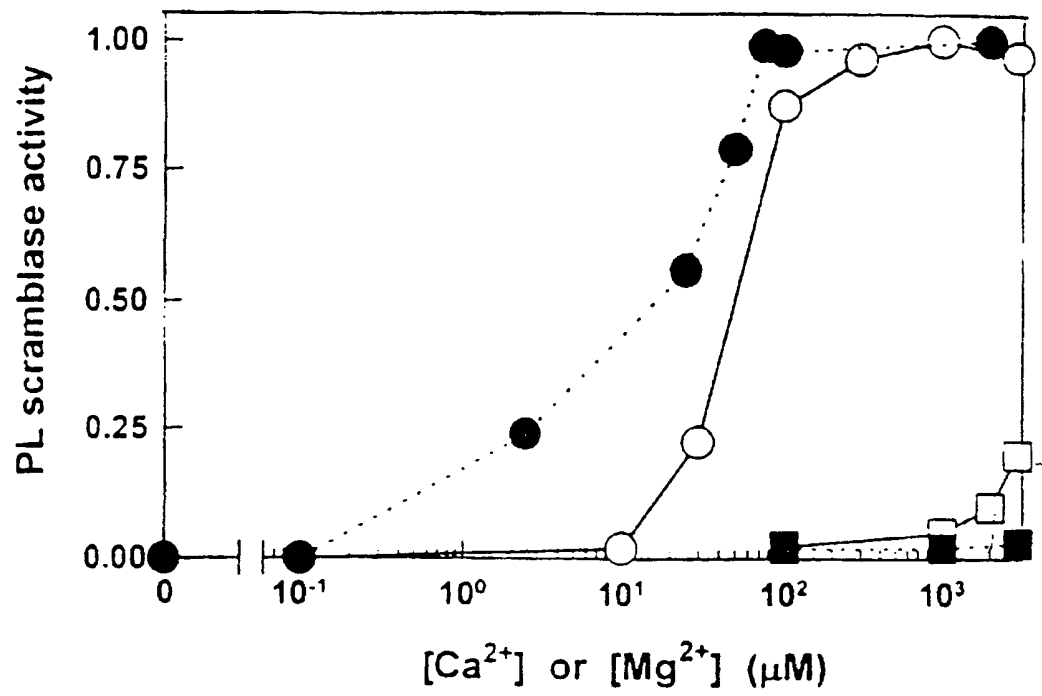having cells of claim 42 attached thereto.

47. The prosthesis of claim 46, wherein the prothesis is
30 a vascular graft.

FIG 1A

FIG 1B
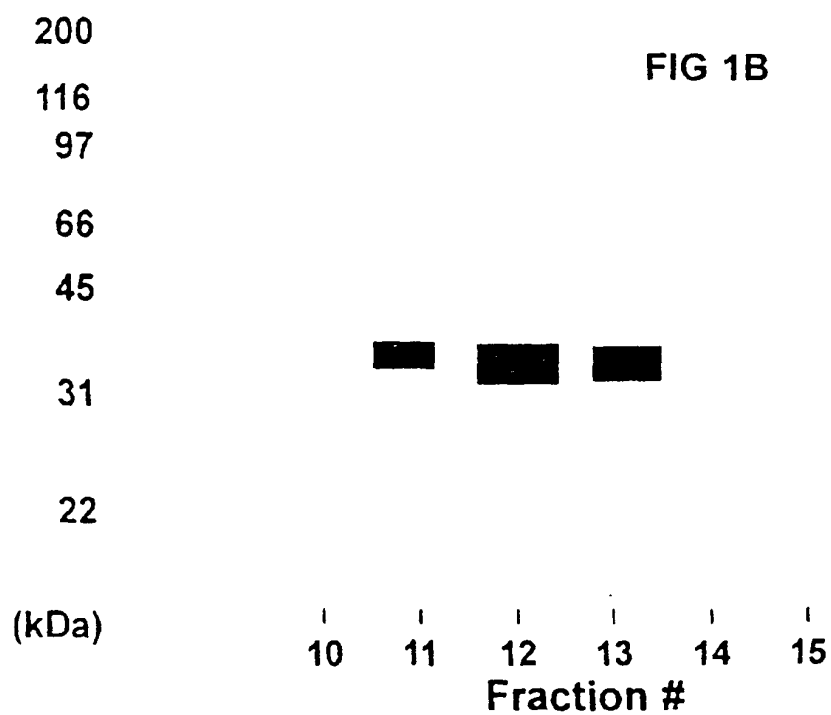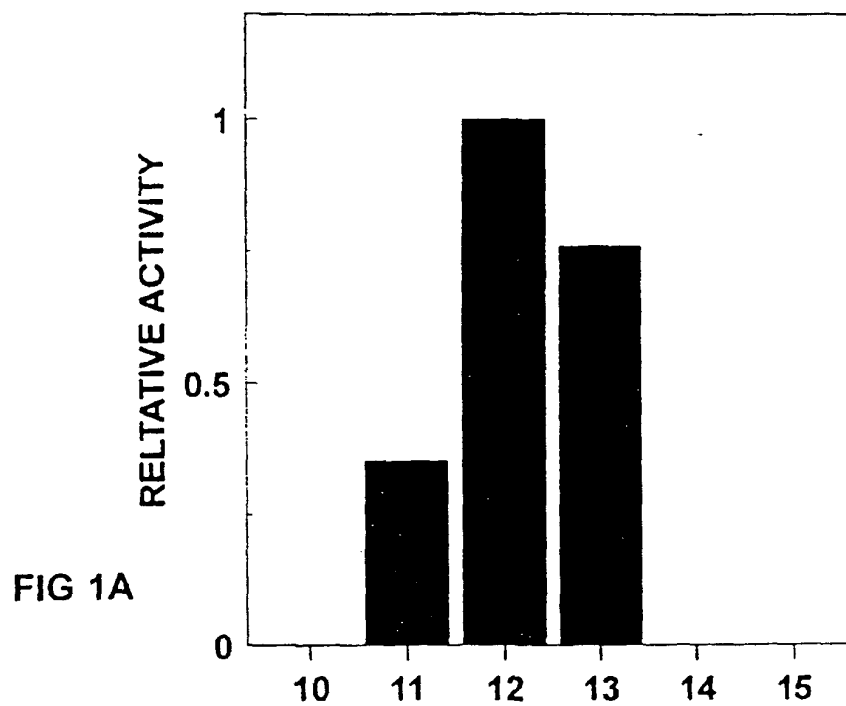
FIG 2
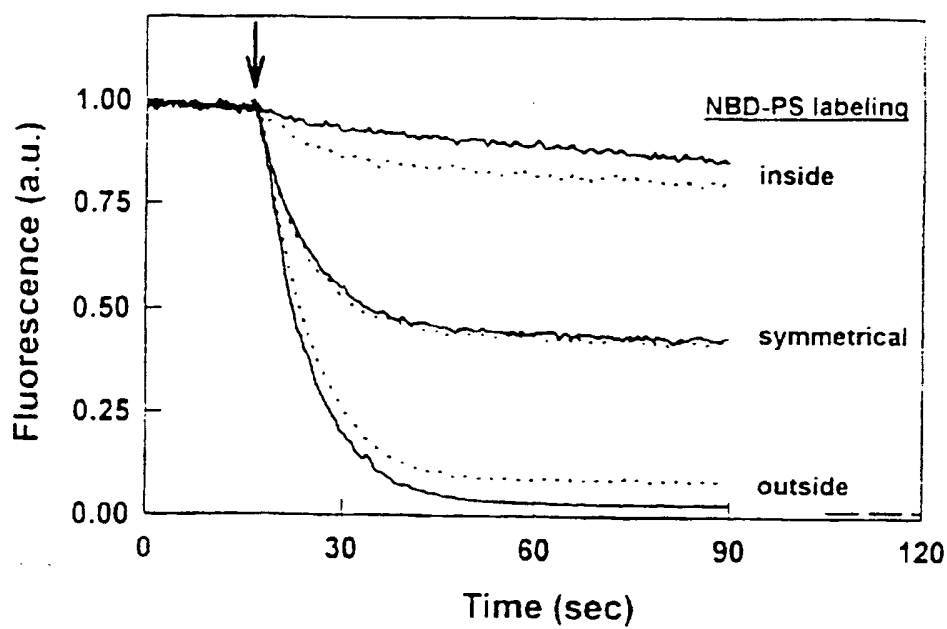
FIG 3

FIG 4

INTERNATIONAL SEARCH REPORT

A.	CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/573; C12N 9/00; C07K 16/00; C07H 21/04
US CL :435/6, 7.4, 13, 183; 530/388.15; 536/23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B.	FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.4, 13, 15, 69.1, 183; 530/350, 388.15, 388.26; 536/23.5
424/9.1, 422, 146.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS, CJACS
Sequence Databases (GENBANK, EMBL, PIR, SWISSPROT, EST, GENESEQ)

C.	DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ---- Y,P	Database, MPSRCH, SMITH-WATERMAN algorithm. Databases n-geneseq26 and EST-STS. May 1997, relevant hits, see entire document.	1-11 ---- 12-31, 33-47
X,P ---- Y,P	BASSE et al. Isolation of an Erythrocyte Membrane Protein that Mediates Ca^{2+}-dependent Transbilayer Movement of Phospholipid. J. Biol. Chem. 19 July 1996, Vol. 271, No. 29, pages 17205-17210. See entire document.	1-11 ---- 12-31, 33-47
X,P ---- Y,P	COMFURIUS et al. Reconstitution of Phospholipid Scramblase Activity from Human Blood Platelets. Biochem. 18 June 1996, Vol. 35, No. 24, pages 7631-7634. See entire document.	1-11 ---- 12-31, 33-47

☒ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier document published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed	"&"	document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
26 MAY 1997	11 JUL 1997
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	Authorized officer DANIEL MYTELKA
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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KOJIMA et al. Production and Characterization of Transformed B-Lymphocytes Expressing the Membrane Defect of Scott Syndrome. J. Clin. Invest. December 1994, Vol. 94, pages 2237-2244. See entire document.	1-29
Y	SMEETS et al. Calcium-induced transbilayer scrambling of fluorescent phospholipid analogs in platelets and erythrocytes. Biochem. Biophys. Acta. 02 November 1994, Vol. 1195, pages 281-286. See entire document.	1-29
Y	VERHOVEN et al. Mechanisms of Phosphatidylserine Exposure, A Phagocyte Recognition Signal, on Apoptotic T Lymphocytes. J. Exp. Med. November 1995, Vol. 182, pages 1597-1601. See entire document.	12-30, 33-34
Y	DEVAUX et al. Maintenance and consequences of membrane phospholipid asymmetry. Chem. Phys. Lipids. 06 September 1994, Vol. 73, pages 107-120. See entire document.	23-29
Y	Database MEDLINE on STN, No. 90158550, ZWAAL et al. Loss of membrane phospholipid asymmetry during activation of blood platelets and sickled cells; mechanisms and physiological significance. Mol. Cell. Biochem. 23 November-23 December 1989, Vol. 1-2, pages 23-31. Abstract.	31

INTERNATIONAL SEARCH REPORT

	international application No. PCT/US97/05198

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
   because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

   Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
   1-31 and 33-47

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest   ☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)*

INTERNATIONAL SEARCH REPORT

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING

This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.

Group I, claim(s) 1-11, drawn to phospholipid scramblase protein and DNA encoding same.

Group II, claim(s) 12-22, drawn to inhibitors of scramblases.

Group III, claim(s) 23-29, drawn to uses of inhibitors to prevent surface exposure of phospholipids in stored red blood cells or platelets.

Group IV, claim(s) 30, drawn to uses of inhibitors to prolong graft survival.

Group V, claim(s) 31, drawn to uses of inhibitors to prevent coagulation of cells in a sickle cell patient.

Group VI, claim(s) 32, drawn to uses of inhibitors to prolong red blood cell lifespan in vivo.

Group VII, claim(s) 33-34, drawn to uses of inhibitors to treat autoimmune diseases.

Group VIII, claim(s) 35-41, drawn to methods of diagnosing patients with abnormal capacities for clot formation.

Group IX, claim(s) 42-47, drawn to methods of engineering cells for transplantation.

The inventions listed as Groups I-IX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I and Group II are drawn to separate products that do not necessarily have anything in common, either structurally or functionally. For example, the scramblases of Group I are Ca^{++} dependent, so they would be inhibited by any compound that chelates divalent cations, such as EDTA or citric acid. Groups III-VII are drawn to five separate uses of the inhibitors. Group VIII includes methods of diagnosis that do not necessarily use the first named product (they might instead use antibodies or coagulation tests). Group IX includes methods of engineering cells that do not necessarily involve any direct changes in the compounds of group I, such as the addition of nucleic acids coding for an inhibitor.